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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/47, A61K 48/00, 38/17

A1

(11) International Publication Number: WO 95/25796

(43) International Publication Date: 28 September 1995 (28.09.95)

(21) International Application Number: PCT/US95/03680

(22) International Filing Date: 23 March 1995 (23.03.95)

(30) Priority Data:
08/216,971 23 March 1994 (23.03.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/216,971 (CIP)
Filed on 23 March 1994 (23.03.94)

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(81) Designated States: AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: C-TERMINALLY TRUNCATED CFTR GENES AND PROTEINS

(57) Abstract

Provided are novel DNA molecules that include a nucleotide sequence encoding novel truncated CFTR polypeptides that have functional Cystic Fibrosis transmembrane conductance regulator (CFTR) protein biological activity. Also provided are the novel truncated CFTR polypeptides that possess the functional CFTR biological activity. The novel polypeptides act as regulated epithelial cell anion (Cl^-) channels.

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Description

C-terminally truncated CFTR genes and proteins

SPECIFICATION

This is a continuation-in-part of copending U.S. Application Serial No. 08/216,971 filed in the U.S. Patent and Trademark Office on 23 March 1994, which is incorporated by reference herein. The invention was made in part under the auspices of a grant from the U.S. Government, who has certain rights in the invention.

BACKGROUND OF THE INVENTION

Cystic Fibrosis (CF) is the most common fatal genetic disease in Caucasians (Boat, T.F. et al. in The Metabolic Basis of Inherited Diseases (Scriver, C.R. et al. eds., McGraw-Hill, New York (1989))). Approximately one in every 2,500 Caucasian infants in the United States is born with the disease. At the present time, there are approximately 30,000 CF patients in the United States. Despite current standard therapy, the median age of survival for CF patients is only 26 years. Disease of the pulmonary airways is the major cause of morbidity and is responsible for 95% of the mortality. The first manifestation of lung disease is often a cough, followed by progressive dyspnea. Tenacious sputum becomes

purulent due to colonization of bacteria. Chronic bronchitis and bronchiectasis can be partially treated with current therapy, *e.g.*, DNAase and antibiotics, but the course is punctuated by increasingly frequent exacerbations of the pulmonary disease. As the disease progresses, the patient's activity is progressively limited. End-stage lung disease is heralded by increasing hypoxemia, pulmonary hypertension, and cor pulmonale.

The upper airways of the nose and sinuses are also involved by CF. Most patients develop chronic sinusitis. Nasal polyps occur in 15-20% of patients and are common by the second decade of life. Gastrointestinal problems are also frequent in CF; infants may suffer meconium ileus. Exocrine pancreatic insufficiency, which produces symptoms of malabsorption, is present in the large majority of patients with CF. Males are almost uniformly infertile and fertility is decreased in females.

Based on both genetic and molecular analyses, a gene associated with CF was isolated as part of 21 individual cDNA clones and its protein product predicted. *See, e.g.*, Kerem, B.S. et al. (1989) *Science* 245:1073-1080; Riordan, J.R. et al. (1989) *Science* 245:1066-1073; Rommens, J.M. et al. (1989) *Science* 245:1059-1065. For example, European patent application

publication number: 0 446 017 A1 describes the construction and expression of the gene, identification of a functional protein product of the gene and confirmation that mutations of the gene are responsible for CF. *See also* Gregory, R.J. et al. (1990) *Nature* 347:382-386; Rich, D.P. et al. (1990) *Nature* 347:358-362.

The protein product of the CF associated gene is called the Cystic Fibrosis transmembrane conductance regulator ("CFTR"). *See, e.g.,* Riordan, J.R. et al. (1989) *Science* 245:1066-1073. Wild-type human CFTR, a monomeric protein of approximately 1480 amino acids in length, comprises two repeated structural motifs. Each of these motifs comprises a membrane-spanning domain ("MSD"), containing six transmembrane segments and a nucleotide binding domain (NBD), that contains sequences for interaction with nucleotides. These two MSD-NBD motifs of CFTR, that are structurally similar, but not identical in amino acid sequence to one another, are separated by a large polar region of the protein called the R domain, which contains multiple potential phosphorylation sites.

The MSD-NBD motif of the amino terminal portion of CFTR may be termed the MSD-1-NBD-1 motif, while the second motif may be termed the MSD-2-NBD-2 motif. MSD-1 contains the first through sixth transmembrane sequences of CFTR, while MSD-2 contains the seventh through twelfth

transmembrane sequences. Each NBD contains so-called Walker A and B sequences that are involved in the interaction with nucleotides. *See* Walker, J.E. et al. (1982), *EMBO J.* 1:945-951.

Based on this predicted domain structure, human CFTR is believed to be a member of a class of related membrane proteins called the traffic ATPases (Ames, G.F.L. et al. (1990), *FEMS Microbiol. Rev.* 75:429-446) or the ATP binding cassette ("ABC") transporters (Hyde, S.C. et al. (1990) *Nature* 346:362-365). A general pattern in this family is one in which two MSD-NBD motifs form a functional complex. In many members of the ABC transporter family, individual domains are encoded by separate genes and the functional complex is a multimer composed of several polypeptides, (*see, e.g.*, Mimura, C.S. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:84-88), unlike human CFTR, which is a monomer encoded by a single gene. The proteins of this family include the multi-drug resistance (MDR) or P-glycoprotein, bovine adenyl cyclase, the yeast STE6 protein as well as several bacterial amino acid transport proteins. *See, e.g.*, Riordan, JR. et al. (1989) *Science* 245:1066-1073; Hyde, S.C. et al. (1990) *Nature* 346:362-365. Proteins in this group, characteristically, are involved in pumping small molecules into or out of cells.

CFTR has been postulated to regulate the outward flow of anions, especially chloride ions (Cl^-), from epithelial cells in response to phosphorylation by cyclic AMP-dependent protein kinase or protein kinase C (Riordan, J.R. et al. (1989) *Science* 245:1066-1073; Frizzell, R.A. et al. (1986) *Science* 233:558-560; Welsh, M.J. and Liedtke, C.M. (1986) *Nature* 322:467; Li, M. et al. (1988) *Nature* 331:358-360; Hwang, T-C. et al. (1989) *Science* 244:1351-1353).

Sequence analysis of the CFTR gene of cells of normal individuals and those with CF has revealed a variety of disease causing mutations. *See, e.g.*, Cutting, G.R. et al. (1990) *Nature* 346:366-369; Dean, M. et al. (1990) Cell 61:863:870; and Kerem, B-S. et al. (1989) *Science* 245:1073-1080; Kerem, B-S et al. (1990) *Proc. Natl. Acad Sci.* USA 87:8447-8451. More than 200 different mutations in the CFTR gene are known, all of which have been found in DNA from CF patients. These mutations result in altered amino acids throughout the CFTR protein. *See* Tsui, L.C. et al. (1993) "Mutation Analysis in Cystic Fibrosis," in *Cystic Fibrosis - Current Topics*: Vol. I, Dodge J.A. et al., eds., John Wiley & Sons, pp. 27-44. Interestingly, no mutations have been found in any of the potential CFTR phosphorylation sites.

Population studies have indicated that the most common CF mutation, a deletion of the 3 nucleotides that encode the normally-occurring phenylalanine at residue 508 of the CFTR amino acid sequence (ΔF508), is associated with approximately 70% of the cases of Cystic Fibrosis. This mutation results in the failure of an epithelial cell chloride channel to respond to cAMP. *See* Frizzell R.A. et al. (1986) *Science 233:558-560;* Welsh, M.J. (1986) *Science* 232:1648-1650.; Li, M. et al. (1988) *Nature* 331:358-360; Quinton, P.M. (1989) *Clin. Chem.* 35:726-730. In airway cells, this mutation leads to an imbalance in ion and fluid transport. It is widely believed that this causes abnormal mucus secretion, and ultimately results in pulmonary infection and epithelial cell damage.

Studies on the biosynthesis (Cheng, S.H. et al. (1990) *Cell* 63: 827-834; Gregory, R.J. et al. (1991) *Mol. Cell Biol.* 11:3886-3893) and localization (Denning, G.M. et al. (1992) *J. Cell Biol.* 118:551-559) of CFTR ΔF508, as well as other CFTR mutants, indicate that many CFTR mutant proteins are not processed correctly and, as a result, are not delivered to the plasma membrane where they function. *See* Gregory, R.J. et al. (1991) *Mol. Cell Biol.* 11: 3886-3893. These conclusions are consistent with earlier functional studies which failed to detect cAMP-stimulated chloride channels in cells expressing

CFTR ΔF508. *See* Rich, D.P. et al. (1990) *Nature* 347:358-363; Anderson, M.P. et al. (1991) *Science* 251:679-682.

To date, the primary objectives of treatment for CF have been to control infection, promote mucus clearance, and improve nutrition (Boat, T.F. et al. in *The Metabolic Basis of Inherited Diseases* (Scriver, C.R. et al. eds., McGraw-Hill, New York (1989)). Intensive antibiotic use and a program of postural drainage with chest percussion have been the mainstays of therapy. However, as the disease progresses, frequent hospitalizations are required. Nutritional regimens include pancreatic enzymes and fat-soluble vitamins. Bronchodilators are used at times. Corticosteroids have been used to reduce inflammation in patients, but they may produce significant adverse effects and their benefits are not certain. In extreme cases, lung transplantation has sometimes been attempted. *See, e.g.*, Marshall, S. et al. (1990) *Chest* 98:1488.

Most efforts to develop new therapies for CF have focused on the pulmonary complications. Because CF mucus consists of a high concentration of DNA, derived from lysed neutrophils, one approach has been the development of recombinant human DNase to breakdown the DNA, which reduces the viscosity of the mucus. *See, e.g.*, Shak, S. et al. (1990) *Proc. Natl. Sci. Acad USA*

87:9188. Aerosolized DNase, known commercially as Pulmozyme, which has been approved for clinical use in the United States, has shown effectiveness in reducing the viscosity of mucus in the lungs and in clearing the airways of obstruction and, perhaps, in reducing infections.

Also, in an attempt to limit damage caused by an excess of neutrophil-derived elastase, protease inhibitors have been tested. For example, alpha-1-antitrypsin purified from human plasma has been aerosolized to deliver enzyme activity to lungs of CF patients. *See, e.g.*, McElvaney, N. et al. (1991) *The Lancet* 337:392. Another approach would be the use of agents to inhibit the action of oxidants derived from neutrophils. Although biochemical parameters have been successfully measured, the long term beneficial effects of these treatments have not been established.

Based on knowledge of the human Cystic Fibrosis gene, three general corrective approaches (as opposed to therapies aimed at ameliorating the symptoms) are currently being pursued to reverse the abnormally decreased chloride secretion and increased sodium absorption in CF airways. Defective electrolyte transport by airway epithelia is thought to alter the composition of the respiratory secretions and mucus. *See, e.g.*, Boat, T.F. et al. in The Metabolic Basis of

Inherited Diseases (Scriver, C.R. et al. eds.), McGraw-Hill, New York (1989); Quinton, P.M. (1990) *FASEB J.* 4:2709-2717). Hence, in a first approach, pharmacological treatments aimed at correcting the abnormalities in electrolyte transport are being pursued. Trials are in progress with aerosolized versions of the drug amiloride; a diuretic that inhibits sodium channels, thereby inhibiting sodium absorption. Initial results indicate that the drug is safe and suggest a slight change in the rate of disease progression, as measured by lung function tests. *See, e.g.*, Knowles, M. et al. (1990) *N. Eng.J. Med* 322:1189-1194; App, E. (1990) *Am. Rev. Respir. Dis. 141-605.* Nucleotides, such as ATP or UTP, stimulate purinergic receptors in the airway epithelium. As a result, they open a class of chloride channel that is different from the CFTR chloride channels. *In vitro* studies indicate that ATP and UTP can stimulate chloride secretion. *See* Knowles, M. et al. (1991) *N. Eng. J. Med.* 325-533. Preliminary trials to test the ability of nucleotides to stimulate secretion *in vivo,* and hopefully correct the electrolyte transport abnormalities are also being performed.

A second approach to curing Cystic Fibrosis, "protein replacement" seeks to deliver functional, recombinant CFTR to CF mutant cells to directly augment the missing CFTR activity. The concept of protein

replacement therapy for CF is simple: a preparation of highly purified recombinant CFTR, formulated in some fusogenic liposome or reassembled virus carrier, is delivered to the cells of the airways by instillation or aerosol. However, attempts at formulating a CF protein replacement therapeutic have met with difficulties. For example, CFTR is not a soluble protein, like those that have been used for previous protein replacement therapies or for other therapeutic uses, *e.g.*, insulin or human growth hormone. Moreover, there may be a limit to the amount of a membrane protein with biochemical activity that can be expressed in a recombinant cell. There are reports in the literature that 10^5-10^6 molecules/cell representing the upper limit (H-Y Wang et al. (1989) *J. Biol. Chem.* 264:14424), compared with about 2000 molecules/second/cell being reported for secreted proteins such as EPO, insulin, growth hormone, and tPA.

In addition to limited expression capabilities, the purification of the full length CFTR, a membrane bound protein containing numerous hydrophobic amino acid residues (especially in the MSDs), is more difficult than purification of a soluble protein. Membrane proteins require solubilization in detergents. Purification of full length CFTR, in the presence of detergents, represents a less efficient process than the purification process required of soluble proteins. Other potential

obstacles to a protein replacement approach include: 1) the inaccessibility of airway epithelium caused by mucus build-up and the hostile nature of the environment in CF airways; 2) potential immunogenicity ; and 3) the fusion of CFTR with recipient cells may be an inefficient.

A third approach to Cystic Fibrosis treatment involves gene therapy, in which DNA encoding Cystic Fibrosis is transferred to CF defective cells (e.g. of the respiratory tract), where it can be expressed. However, most current methods to introduce DNA into cells are generally inefficient. Since viruses have evolved very efficient means to introduce their nucleic acid into cells, approaches to gene therapy often make use of engineered defective viruses, such as adenovirus or retroviruses. However, viral vectors have limited space for accommodating foreign genes. For example, although adeno-associated virus, (AAV), is an attractive gene therapy vector in many respects, it can only accommodate 4.5 Kb of exogenous DNA and still be properly packaged. DNA encoding full length wild-type CFTR is at least 4.5 kb and thus represents the upper packaging size limit. Thus, it is believed that gene therapy approaches to CF will face many of the same clinical challenges as protein therapy.

Although there has been notable progress in developing curative therapies for CF, based on knowledge

of the gene encoding CFTR, the expressed protein product and mechanism of action, there are significant obstacles confronting every approach. New therapies for CF are needed.

SUMMARY OF THE INVENTION

The present invention offers new therapies for treating Cystic Fibrosis, that are based on novel DNA molecules and polypeptides encoded thereby. The present invention is based on the novel finding that a truncated wild-type human CFTR polypeptide that does not contain a significant portion of the carboxy terminal amino acid sequence of CFTR, including the MSD-2 and NBD-2 domains, forms a regulated anion channel in host cells expressing a DNA molecule that includes a nucleotide sequence coding for the novel polypeptide. For reference, in the parent application, U.S. Application Serial No. 08,216,971, this polypeptide was referred to as a "CF Monomer." For purposes of clarifying the invention, the polypeptide of the invention is now referred to as a truncated CFTR polypeptide.

Thus, in one aspect, the present invention provides a DNA molecule that includes a nucleotide sequence encoding a truncated Cystic Fibrosis transmembrane conductance regulator (CFTR) polypeptide that lacks a significant portion of the carboxy terminal

amino acid sequence of wild-type human CFTR, but possesses a biological activity significantly duplicative of wild-type human CFTR so as to function as a regulated epithelial cell anion (*e.g.*, Cl^-) channel. In a preferred embodiment, the DNA molecule encodes a truncated CFTR polypeptide that includes the amino terminal sequence of human CFTR, including at least the MSD-1 domain, the NBD-1 domain and R domain, and which provides a functional chloride ion channel and a regulator of the opening and closing of the chloride channel. In a more preferred embodiment, the DNA molecule comprises the D836X construct which includes a nucleotide sequence that encodes the amino terminal 836 amino acids of human CFTR that includes the MSD-1, NBD-1 and R domains. In an especially preferred embodiment, the DNA molecule has the nucleotide sequence set forth as SEQ ID NO. 1.

The invention also contemplates the aforementioned DNA molecules encoding truncated CFTR polypeptides that contain alterations or modification in nucleotide sequences that can produce an expression product having a modified or altered amino acid sequence, such modifications or alterations being limited to those that do not abolish or destroy the regulated anion (*e.g.*, Cl^-) channel biological activity of the expressed polypeptide.

Further aspects of the invention include methods for making and expressing the disclosed DNA molecules, as well as preferred constructs and delivery vehicles containing the DNA molecules for use in gene therapies for CF.

In another aspect, the invention also relates to the truncated CFTR polypeptides *per se* that lack a significant portion of the carboxy terminal amino acid sequence of wild-type human CFTR, but which possesses a biological activity sufficiently duplicative of wild-type human CFTR so as to function as a regulated epithelial cell anion (Cl^-) channel. Thus, such truncated CFTR polypeptide molecules comprise a chloride ion channel and a regulator of the opening and closing of the channel. In a preferred embodiment, the truncated CFTR polypeptide includes the membrane spanning domain (MSD-1), the nucleotide binding domain-1 (NBD-1) and the R domain of human CFTR. In a more preferred embodiment, the truncated CFTR polypeptide is the expression product of the D386X DNA construct and contains the amino terminal 836 amino acid residues of human CFTR. In an especially preferred embodiment, the truncated CFTR polypeptide has the amino acid sequence set forth as SEQ ID NO. 2.

The invention also contemplates the aforementioned truncated CFTR polypeptide molecules that may contain amino acids substitutions or alterations not

found in wild-type human CFTR, so long as such amino acid substitutions or alterations do not abolish or destroy the regulated anion (Cl^-) channel biological activity of the truncated CFTR polypeptide.

Further aspects of the instant invention include methods for making the disclosed polypeptides, as well as preferred delivery vehicles for performing CF protein replacement therapies.

The DNA molecules of the invention include a nucleotide sequence encoding the truncated CFTR polypeptides of the invention, in particular, those including the MSD-1, NBD-1 and R domains, is at least 40% shorter than the nucleotide sequence encoding full length CFTR (circa 4.5 +kb). Thus, the DNA molecules of the invention are better accommodated by available gene therapy vectors useful for gene therapy of Cystic Fibrosis. In addition, DNA molecules that include a nucleotide sequence encoding a CFTR polypeptide can be more easily expressed than full-length CFTR in host cells harboring the DNA molecule, inserted therein using a viral vector or lipid vehicle. Moreover, host cells containing the recombinant vector or lipid vehicle can produce significant amounts of truncated CFTR polypeptide in cells expressing the DNA coding for the truncated CFTR polypeptide. Further, the expressed truncated CFTR polypeptide may be more soluble and therefore are more

readily purified from host cells than full length CFTR. These advantages and more make the novel DNA molecules and truncated CFTR polypeptide molecules of the invention attractive as gene therapy and protein replacement therapeutics, respectively, for Cystic Fibrosis patients.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be better understood by reference to the accompanying drawings of which

Fig. 1 is an autoradiograph of polyacrylamide gel electrophoresis showing expression of D836X in HeLa Cells.

Fig. 2 provides a comparison of D836X expression product and of Wild-Type CFTR Cl^-channel activity before and after PKA-dependent phosphorylation; (A) represents single channel recordings from excised inside-out membrane patches from HeLa cells; (B) depicts P_o values before and after PKA - dependent phosphorylation.

Fig. 3 shows the effect of intracellular MgATP concentration on D836X expression product Cl^-channel activity following PKA - dependent phosphorylation.

Fig. 4 shows that intracellular ADP inhibits D836X expression product Cl^-channels.

Fig. 5 provides the sedimentation patterns of the D836X expression product and wild-type CFTR on

sucrose gradient centrifugation; (A) is an autoradiogram of the D836X expression product; (B) is an autoradiogram of CFTR; (C) is a graph showing the quantitative distribution of the D836X expression product and of CFTR in the sucrose gradients.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel DNA molecules and polypeptides encoded thereby that offer new therapeutic possibilities for treating Cystic Fibrosis.

For purposes of better understanding of the present invention, the following definitions are provided:

Unless otherwise indicated, "*polypeptide*" shall include protein, polypeptide and/or peptide.

"CFTR or Cystic Fibrosis Transmembrane Conductance Regulator protein"- refers to a 1480 amino acid monomeric protein. Amino acid sequence analysis suggests that the human CFTR protein comprises two motifs, each motif containing a membrane-spanning domain ("MSD") and a nucleotide binding domain ("NBD"), linked by a unique R domain, that functions as an epithelial cell anion (e.g., Cl^-) channel, regulated by phosphorylation and by nucleoside triphosphates.

The phrase *"cystic fibrosis transmembrane conductance regulator (CFTR) activity or function"*- is

meant to refer to biological functions normally performed by wild-type CFTR. Such functions include, *inter alia*, mediation of anion (especially chloride ion) transport across cellular membranes, particularly those of epithelial cells. Thus, the functional biological activity of CFTR is as a regulated anion (Cl^-) channel.

A "Cystic Fibrosis (CF) defective cell" is a cell that contains a mutant CFTR and lacks wild type CFTR activity or function. There are at least 200 known mutations in the CFTR gene that produce a mutant CFTR lacking wild-type CFTR activity or function. *See, e.g.*, Tsui, L-C (1992) "The Spectrum of Cystic Fibrosis Mutations," *Trends in Genetics* 8 (11) 329-398.

"Truncated CFTR polypeptide" as used herein means a polypeptide that includes the amino terminal portion of the human CFTR amino acid sequence, but lacks a significant portion of the carboxyl terminal amino acid sequence of human CFTR, including MSD-2 and NBD-2, and possesses the biological activity of CFTR, *i.e.*, acts as an epithelial cell anion (Cl^-) channel regulator. In particular, a truncated CFTR polypeptide that exhibits CFTR activity includes the MSD-1, NBD-1 and R domains of CFTR. For reference, in the parent application, U.S. Application Serial No. 08/216,971, this polypeptide was referred to as "CF Monomer." For further clarity in

defining the invention, the polypeptide is referred to as truncated CFTR polypeptide.

"R (regulator) domain" refers to the domain in CFTR that keeps the anion (Cl^-) channel closed at rest and which opens the channel when phosphorylated (e.g. by cAMP-dependent protein kinase (PKA) or protein kinase C (PKC)). The R domain of CFTR , which, thus, regulates anion passage through the Cl^- channel, is encoded by exon 13 of the genomic CFTR gene, and includes a 241 amino acid sequence spanning from about amino acid residue 590 to residue 830 of full length CFTR.

"NBD or Nucleotide Binding Domain" refers to one of two domains in CFTR that binds nucleotide triphosphates (*e.g.*, adenosine triphosphate (ATP)). *NBD-1 or Nucleotide Binding Domain-1* refers to the amino terminal nucleotide binding domain of CFTR, including an amino acid sequence that spans from about amino acid residue 360 to residue 708 of full length CFTR.

"MSD or Membrane Spanning Domain" refers to one of two domains in CFTR that form an epithelial cell anion (Cl^-) chloride channel. Each MSD includes six transmembrane segments of the polypeptide that form the chloride ion channel. *"MSD-1 or Membrane Spanning Domain-1"* refers to the amino terminal membrane spanning domain of CFTR that includes an amino acid sequence that

spans from about amino acid residue 76 to residue 360 of CFTR.

"CF gene therapy" refers to the transfer of a DNA molecule that includes a nucleic acid sequence encoding a protein or polypeptide having functional CFTR activity into a host to treat or prevent Cystic Fibrosis ("CF").

"CF protein replacement therapy" refers to transfer of a protein or polypeptide having functional CFTR activity into a host to treat or prevent CF.

The present invention is based on the novel finding that a truncated CFTR polypeptide that does not contain a significant portion of the carboxy terminal amino acid sequence of wild-type human CFTR, including the MSD-2 and NBD-2 domains, forms a regulated anion channel in host cells expressing a DNA molecule that includes a nucleotide sequence coding for the novel polypeptide.

Thus, in one aspect, the present invention provides a DNA molecule that includes a nucleotide sequence encoding a truncated Cystic Fibrosis transmembrane conductance regulator (CFTR) polypeptide that lacks a significant portion of the carboxy terminal amino acid sequence of wild-type human CFTR, but possesses a biological activity significantly duplicative of wild-type CFTR so as to function as a regulated

epithelial cell anion (*e.g.*, Cl^-) channel. In a preferred embodiment, the DNA molecule encodes a truncated human CFTR polypeptide that includes the amino terminal sequence of CFTR, including at least the MSD-1 domain, the NBD-1 domain and R domain, and which provides a functional chloride ion channel and a regulator of the opening and closing of the chloride channel. In a more preferred embodiment, the DNA molecule comprises the D836X construct which includes a nucleotide sequence that encodes the amino terminal 836 amino acids of human CFTR that includes the MSD-1, NBD-1 and R domains. In an especially preferred embodiment, the DNA molecule has the nucleotide sequence set forth as SEQ ID NO. 1.

The invention also contemplates the aforementioned DNA molecules encoding truncated CFTR polypeptides that contain alterations or modification in nucleotide sequences that can produce an expression product having a modified or altered amino acid sequence, such modifications or alterations being limited to those that do not abolish or destroy the regulated anion (*e.g.*, Cl^-) channel biological activity of the expressed polypeptide.

In another aspect, the invention also relates to the truncated CFTR polypeptides *per se* that lack a significant portion of the carboxy terminal amino acid sequence of wild-type human CFTR, but which possesses a

biological activity sufficiently duplicative of wild-type human CFTR so as to function as a regulated epithelial cell anion (Cl^-) channel. Thus, such truncated CFTR polypeptide molecules comprise a chloride ion channel and a regulator of the opening and closing of the channel. In a preferred embodiment, the truncated CFTR polypeptide includes the membrane spanning domain (MSD-1), the nucleotide binding domain-1 (NBD-1) and the R domain of human CFTR. In a more preferred embodiment, the truncated CFTR polypeptide is the expression product of the D386X DNA construct and contains the amino terminal 836 amino acid residues of CFTR. In an especially preferred embodiment, the truncated CFTR polypeptide has the amino acid sequence set forth as SEQ ID NO. 2.

The invention also contemplates the aforementioned truncated CFTR polypeptide molecules that may contain amino acids substitutions or alterations not found in wild-type human CFTR, so long as such amino acid substitutions or alterations do not abolish or destroy the regulated anion (Cl^-) channel biological activity of the truncated CFTR polypeptide.

Further aspects of the invention include methods for making and expressing the disclosed DNA molecules, as well as preferred constructs and delivery vehicles containing the DNA molecules for use in gene therapies for CF.

Additional aspects of the instant invention include methods for making the disclosed polypeptides, as well as preferred delivery vehicles for performing CF protein replacement therapies.

Thus, the DNA molecules of the invention that include a nucleotide sequence encoding the truncated CFTR polypeptides of the invention, in particular, those including the MSD-1, NBD-1 and R domains, is at least 40% shorter than the nucleotide sequence encoding full length CFTR (circa 4.5 +kb). It is believed that the DNA molecules of the invention are better accommodated by available gene therapy vectors useful for gene therapy of Cystic Fibrosis. In addition, DNA molecules that include a nucleotide sequence encoding a CFTR polypeptide can be more easily expressed than full-length CFTR in host cells harboring the DNA molecule, inserted therein using a viral vector or lipid vehicle. Moreover, host cells containing the recombinant vector or lipid vehicle can produce significant amounts of truncated CFTR polypeptide in cells expressing the DNA coding for the truncated CFTR polypeptide. Further, the expressed truncated CFTR polypeptide may be more soluble and therefore are more readily purified from host cells than full length CFTR. These advantages and more make the novel DNA molecules and truncated CFTR polypeptide molecules of the invention

attractive as gene therapy and protein replacement therapeutics, respectively, for Cystic Fibrosis patients.

Methods For Obtaining a DNA Molecule Including a Nucleotide Sequence Encoding A Truncated CFTR Polypeptide Having Activity as a Regulated Chloride Ion Channel

The nucleotide and amino acid sequence for full-length wild-type human CFTR and the many already determined genetic mutations resulting in non-functional CF mutants are known in the art, *see, e.g.*, European patent application publication number: 0 446 017 A1, incorporated herein by reference. As previously stated, there are over 200 different mutations throughout the CFTR gene that are known to result in a mutant CFTR protein and disease. *See, e.g.*, Tsui et al., "Mutation Analysis in Cystic Fibrosis," 1993, in *Cystic Fibrosis - Current Topics: Volume I*, edit. by Dodge et al., John Wiley & Sons, pp. 27-44.

Based on this information, one of skill in the art can obtain a DNA molecule that includes a nucleotide sequence encoding a truncated CFTR polypeptide having the biological activity of CFTR protein, (*i.e.*, functions as a regulated chloride ion channel) using techniques that are well-known. General references, known to those skilled in the art, that provide numerous techniques useful in the practice of the present invention include, *inter alia*, *Current Protocols in Molecular Biology*,

Ausubel, F. et al., eds., Wiley and Sons, New York; *Molecular Cloning, A Laboratory Manual*, 2d Ed., Sambrook, J. et al., eds., Cold Spring Harbor Laboratory Press, New York (1989); *Methods in Enzymology* (various volumes), Academic Press, New York.

For example, genomic DNA or cDNA encoding human CFTR can be isolated from appropriate cells or plasmids using known standard techniques (e.g. restriction enzyme cleavage). DNA molecules encoding full-length CFTR can then be modified (e.g. via site-directed mutagenesis) to obtain a DNA molecule that contains a nucleotide sequence coding for truncated CFTR having the biological activity of CFTR, which can include the MSD-1, NBD-1 and R domains. Alternatively, an appropriate DNA molecule of the invention can be generated synthetically using standard modes of polynucleotide synthesis. A candidate DNA molecule can be readily tested to determine whether it, in fact, encodes a suitable truncated CFTR polypeptide that has functional CFTR activity, for example, using the SPQ assay disclosed in detail in Example 3, below.

An "expression cassette" comprising the DNA molecule encoding the truncated CFTR polypeptide operably linked to or under the control of transcriptional and translational regulatory elements (e.g. a promoter, ribosome binding site, operator, or enhancer) can be made

and used for expression of CF protein monomers *in vitro* or *in vivo*. The choice of regulatory elements employed may vary, depending for example on the host cell to be transfected and the desired level of expression. Several promoters for use in mammalian cells are known in the art and include, *inter alia*, the phosphoglycerate (PGK) promoter, the simian virus 40 (SV 40) early promoter, the Rous sarcoma virus (RSV) promoter, the adenovirus major late promoter (MLP) and the human cytomegalovirus (CMV) immediate early I promoter. However, any promoter that facilitates suitable expression levels for production of suitable levels of functional chloride ion channels in host cells can be used in the invention. Inducible promoters, (e.g. those obtained from the heat shock gene, metallothionein gene, beta interferon gene, or steroid hormone responsive genes) may be useful for regulating transcription based on external stimuli.

Other regulatory elements known to those skilled in the art that may be used to enhance expression of DNA encoding a truncated CFTR polypeptide having biological activity as a regulated epithelial cell Cl^-channel include, *inter alia*, enhancer elements, such as the CMV enhancer or the RSV enhancer and translational enhancing sequences, such as mRNA capping sequences, Kozak sequences and polyadenylation sequences, including, *inter alia*, the bovine growth hormone (BGH) poly A or the

SV40 early poly A. Other regulatory sequences that may be used to enhance expression of functional chloride ion channels in the present invention can involve the use of specific introns in DNA sequences or the use of mini genes instead of cDNA.

A particularly preferred DNA molecule of the invention includes a nucleotide sequence encoding a truncated CFTR polypeptide that includes MSD-1, NBD-1 and R domains of wild-type CFTR. Such a DNA molecule includes nucleotides 1-2638 of the full length CFTR gene, which sequence is set forth as SEQ ID NO. 1. The DNA of SEQ ID NO. 1 encodes an 836 amino acid fragment of the full length 1480 amino acid CFTR protein, derived from the amino terminal portion of human CFTR, that includes the MSD-1, NBD-1 and R domains. The amino acid sequence of this truncated CFTR polypeptide is set forth as SEQ ID NO. 2. Methods for obtaining this DNA molecule are described in more detail in Example 1. The DNA molecules of the invention may be further modified, for example, to provide for increased stability of the expressed polypeptide product, by the addition of a nucleotide sequence that codes for a carboxyl terminal moiety from human CFTR or another related membrane protein. Other possible alterations or modifications that may result in increased stability of the expressed truncated CFTR polypeptide are discussed below (Example 8).

Those skilled in the art would understand that additional alterations or substitutions in the nucleotide sequence of the DNA molecules of the invention are possible in order to encode truncated CFTR polypeptide molecules within the scope of the present invention. The primary limits on such changes are to not produce a truncated CFTR polypeptide that has had its biological activity of being a regulated Cl^-channel abolished or destroyed. Thus, known mutations that are present in the CFTR molecules of CF patients are avoided. However, as set forth *infra*, there are several groups of changes in the truncated CFTR polypeptides (and the DNA molecules coding therefor) that are possible and within the ability of those skilled in the art. The SPQ assay of Example 3 may be readily and easily used to test the functional Cl^- channel activity of such altered truncated CFTR polypeptides to determine which are active and useful.

Additional modifications to the polypeptide may be made which might increase its function. For example, mutations in the NBD that affect ATP binding and/or hydrolysis might make a channel which has an open state probability greater than that of wild-type human CFTR. For example, it has been shown that the mutation P574H, in NBD-1, produced a channel with an open state probability greater than that of wild-type. *See, e.g.*, Sheppard, D. et al. (1995), *EMBOJ*. 14:101-109. This or

other mutations in these domains which increase the open time of a single channel molecule could be combined with the truncation in constructs, *e.g.*, related to D836X.

Mutations in the R domain which might increase channel function could also be made. For example, it has been shown that deletion of part of the R domain produced a channel which was open even without phosphorylation by cAMP-dependent protein kinase. *See, e.g.*, Rich, D.P. et al. (1991), *Science* 253:205-207. Mutations to the R domain that would increase channel activity by substituting serines that are phosphorylated with negatively charged aspartate may be made. It has been previously shown that such mutations generate a channel that is open even without phosphorylation. *See, e.g.*, Tsui, L.C. et al. (1992), *Human Mutation* 1:197-203.

Additional mutations in the membrane spanning domain might produce a channel which remains Cl^-selective but which has a greater single channel conductance than does wild-type human CFTR. Attempts to identify such a channel would involve site-directed mutations located in the transmembrane sequences followed by their study with the single-channel patch-clamp technique (Example 2), the SPQ halide efflux assay (Example 3), or expression in epithelia with measurement of the Cl^-secretory current. By increasing the flow of Cl^-through single molecules, a

channel which had greater than wild-type function could be generated.

A number of other mutations are also possible which might have little functional consequence on CFTR. For example, there are a number of mutations known as polymorphisms in the coding region of the CFTR gene that do not cause disease. These polymorphisms can occur throughout the coding sequence. *See, e.g.*, Tsui, L.C. et al. (1992), *Human Mutation* 1:197-203. It is expected that a number of such mutations would have no functional consequences for channel function.

It is also possible that deletion of sequences before the first membrane spanning domain in the N-terminal cytoplasmic tail might have little consequence on CFTR Cl^-channel function.

Methods For Making Truncated CFTR Polypeptides

A DNA molecule that includes a nucleotide sequence coding for a truncated CFTR polypeptide, as above, *e.g.*, one that contains the MSD-1, NBD-1 and R domains of human CFTR (preferably in a suitable expression cassette), can be introduced into suitable host cells in culture using standard techniques (e.g. via calcium phosphate or calcium chloride co-precipitation, DEAE dextran mediated transfection, lipofection, or electroporation). Recombinant cells containing the

introduced DNA molecule can then be cultured *in vitro* in a manner that allows expression and functional activity of the truncated CFTR polypeptide. Preferred host cells for generating CF protein monomers include, *inter alia*, mammalian cells, such as COS and C127; yeast cells and insect cells.

The truncated CFTR polypeptides of the invention are integral membrane proteins (they contain MSD-1, which comprises six transmembrane segments of the protein that forms the chloride ion channel) that can be purified from host cell membranes using known protein purification methods, such as ion exchange chromatography, gel filtration chromatography, electrophoresis and affinity chromatography. *See, e.g.*, Tilly et. al. (1992) *The Journal of Biological Chemistry*, Vol 267, No. 14, pp. 9470-73; Anderson et. al. (1991) Science 251, pp. 679-682. A preferred method of purification involves first solubilizing the protein from the membrane in the presence of a nondenaturing detergent, and then using the aforementioned purification techniques to obtain a truncated CFTR polypeptide which, when present in an epithelial cell membrane, functions as a regulated chloride ion channel.

As an alternative to recombinant methods, a truncated CFTR polypeptide according to the invention can also be obtained from CFTR protein, e.g., by enzymatic

cleavage, which results in a product that has epithelial cell anion (Cl^-) channel regulatory activity.

Purified truncated CFTR polypeptides produced as described herein also can be used, for example, as an immunogen to generate antibodies specific to the amino terminal portion of CFTR. In addition, the truncated CFTR polypeptides and DNA molecules coding therefor to precisely map and determine the function of a particular region or domain of CFTR. Moreover, truncated CFTR polypeptides of the invention have use in protein replacement therapies and the DNA molecules coding therefor in gene therapies for treatment of Cystic Fibrosis, as described in detail below.

Protein Replacement Therapy

Protein therapy may be accomplished by any method that effectively introduces a truncated CFTR polypeptide, *e.g.*, one that includes the MSD-1, NBD-1 and R domains, into the membrane of CF defective cells to restore normal CFTR activity in such cells, primarily epithelial cells of the lung. An effective amount of a truncated CFTR polypeptide (*i.e.*, an amount sufficient to reduce or eliminate the symptoms associated with CF) can be administered alone or in association with an agent that facilitates passage (*e.g.*, via fusion or endocytosis) through cell membranes to the effected cells

CF patients (i.e. patients having CF defective cells). The "effective amount" can be determined by one of skill in the art based on such factors as the type and severity of symptoms being treated, the weight and/or age of the subject, the previous medical history of the subject, and the selected route for administration of the agent.

Preferably for use in protein therapy, truncated CFTR polypeptides of the invention are associated with lipids, such as detergents or other amphipathic molecule micelles, membrane vesicles, liposomes, virosomes, or microsomes. Lipid compositions that are naturally fusogenic or can be engineered to become fusogenic (e.g., by incorporating a fusion protein into the lipid) are especially preferred. Fusion proteins can be obtained from viruses such as parainfluenza viruses 1-3, respiratory syncytial virus (RSV), influenza A, Sendai virus, and togavirus fusion protein. Nonviral fusion proteins include normal cellular proteins that mediate cell-cell fusion. Other nonviral fusion proteins include the sperm protein PH-30 which is an integral membrane protein located on the surface of sperm cells that is believed to mediate fusion between the sperm and the egg. *See* Blobel et al. (1992) *Nature* 356:248-251. Still other nonviral fusion proteins include chimaeric PH-30 proteins such as PH-30 and the binding component of hemagglutinin from influenza virus

and PH-30 and a disintegrin (*e.g.*, bitistatin, barbourin, kistrin, and echistatin). In addition, lipid membranes can be fused using traditional chemical fusogens such as polyethylene glycol (PEG).

A CF patient can be treated by administration of an effective amount of a truncated CFTR polypeptide of the invention, optionally in a pharmaceutically acceptable carrier or diluent. An effective amount of a truncated CFTR polypeptide of the invention is an amount sufficient alleviate the symptoms of CF, *i.e.*, an amount sufficient to restore functional CFTR activity to the cells of the patient, and can be determined with routine trial by the clinician. A CF protein monomer can be administered subcutaneously, intravenously, intraperitoneally, intramuscularly, parenterally, orally, submucosally, by inhalation, or other appropriate route of administration in an effective dosage range. A preferred route of administration is by inhalation (*e.g.*, of an aerosolized pharmaceutical composition), *i.e.*, to be directed to the lungs. If necessitated by a particular mode of administration, truncated CFTR polypeptides of the invention can be encapsulated within a material that protects it from enzymatic degradation. In addition, prior to administration, it may be useful to administer agents to clear mucus (*e.g.*, using a DNAse)

and/or bacterial infection, to facilitate delivery to the epithelial cells of the lungs.

Gene Therapy

Alternatively, a preparation of the DNA molecule including a nucleotide sequence encoding a truncated CFTR polypeptide according to the invention can be incorporated into a suitable vector or other vehicle for delivering the gene into a CF patient's CFTR-defective cells. As many of the symptoms of CF manifest themselves in the respiratory tract, the preparation can be delivered directly to the airways of CF patients.

The first generation of CF gene therapy is likely to be transient and to require repeated delivery of effective replacement DNA to the affected cells of the airways. Eventually, however, gene therapy may offer a more permanent therapy or even cure for the disease when the identity of the precursor or stem cell to airway epithelial cells becomes known. Stable incorporation of DNA molecules encoding truncated CFTR polypeptides according to the present invention into airway epithelial stem cells will provide for the production of the truncated CFTR polypeptide by subsequent generations of such epithelial cells under the direction of the integrated DNA sequences and can correct the

physiological defect resulting in CF by providing to such cells functional CFTR biological activity.

For use in treating CF, appropriate vectors must: 1) effectively infect lung epithelia or other tissue manifesting the disease and deliver the therapeutic nucleic acid encoding function CFTR biological activity; 2) be appropriately maintained in host cells; and 3) be safe. The following describes a number of approaches and vectors and vehicles that may prove useful for performing CF gene therapy. The following listing, however, is not intended to be exhaustive and many other vectors or vehicles should prove useful for performing gene therapy with the novel DNA molecules disclosed herein.

Retroviruses - Although defective retroviruses are the best characterized system and so far the only one approved for use in human gene therapy, (*see, e.g.*, Miller, A.D. (1990) *Blood* 76:271), the major issue in relation to CF is the requirement for dividing cells to achieve DNA integration and gene expression. Were conditions found to induce airway cell division, the *in vivo* application of retroviruses, especially if repeated over many years, would necessitate assessment of the safety aspects of insertional mutagenesis in this context.

Adeno-Associated Virus - (AAV) is a naturally occurring defective virus that requires other viruses such as adenoviruses or herpes viruses as helper viruses. *See* Muzyczka, N. (1992) in *Current Topics in Microbiology and Immunology* 15 8:97. It is also one of the few viruses that may integrate its DNA into non-dividing cells, although this is not yet certain. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. AAV vectors, therefore, are useful for expression of a DNA molecule encoding a truncated CFTR polypeptide according to the present invention, because the size of the insert is well within the range that may be accommodated by an AAV vector. On the other hand, AAV is less useful for expressing a DNA molecule which encodes full length CFTR because the size of such a DNA (>4.5 kb) approaches or may exceed AAV's upper limit.

Naked DNA - Naked plasmid can be introduced into muscle cells by injection into the tissue. Expression can extend over many months but the number of positive cells is low. *See, e.g.*, Wolff, J. et al. (1989) *Science 247:1465*.

DNA-Lipid Complexes - Lipid carriers, *e.g.*, liposomes, can be associated with naked DNA (*e.g.*, plasmid DNA) to facilitate passage through cellular

membranes. Cationic, anionic, or neutral lipids can be used for this purpose. However, cationic lipids are preferred because they associate better with DNA, which possesses a net negative charge. Cationic lipids have been shown to mediate intracellular delivery of plasmid DNA. *See, e.g.*, Felgner, P. and Ringold, G.M. (1989) *Nature* 337:387. Injection of cationic lipid plasmid DNA complexes into the circulation of mice has been shown to result in expression of the DNA in lung. *See, e.g.*, Brigham, K. et al. (1989) *Am. J. Med. Sci.* 298:278. Instillation of cationic lipid plasmid DNA into lung has also been found to be expressed in epithelial cells but the efficiency of expression has been reported as being relatively low and transient. *See, e.g.*, Hazinski, T.A. et al. (1991) *Am. J. Respir., Cell Mol. Biol.* 4:206.

Receptor Mediated Entry - One potential means for improving the efficiency of DNA uptake by target cells utilizes receptor-mediated endocytosis as an entry mechanism and protection of DNA in complexes with polylysine. *See, e.g.*, Wu, G. and Wu, C.H. (1988) *J Biol. Chem.* 263:14621. One potential problem with this approach, however, is that the incoming DNA enters the host cell's pathway leading from endosome to lysosome, where much incoming material is degraded. One solution to this problem utilizes transferrin-DNA-polylysine complexes linked to adenovirus capsids. *See, e.g.*,

Curiel, D.T. et al. (1991) *Proc. Natl. Acad Sci. USA* 88:8850. The latter enter efficiently, but have the added advantage of naturally disrupting the endosome thereby avoiding shuttling to the lysosome.

Adenovirus - Defective adenoviruses at present appear to be one of the more promising approaches to effecting CF gene therapy. *See, e.g.*, Berkner, K.L. (1988) *BioTechniques* 6:616. Adenoviruses, which have a trophism for epithelial cells of the airway and lungs, can provide delivery of a DNA molecule encoding CFTR or a truncated CFTR polypeptide having functional regulated chloride ion channel activity to complement the defect in CF patient's cells.

Adenovirus can be manipulated, such that it encodes and expresses the desired gene product, here, truncated CFTR polypeptide having the biological property of epithelial cell chloride ion channel regulation, and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. In addition, as noted, adenovirus has a natural trophism for airway epithelia. The viruses are able to infect quiescent (non-dividing) cells as are generally present in the airways, offering a major advantage over retroviruses. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about

insertional mutagenesis. Furthermore, adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile. *See, e.g.*, Schwartz, A.R. et al. (1974) *Am. Rev. Respir. Dis.* 109:233-238. Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances, including, *inter alia,* transfer of DNA encoding alpha-1-antitrypsin and CFTR to the lungs of cotton rats. *See, e.g.*, Rosenfeld, M.A. et al. (1991) *Science* 252:431-434; Rosenfeld et al., (1992) *Cell* 68:143-155. Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative. *See, e.g.*, Green, M. et al. (1979) *Proc. Natl. Acad Sci. USA* 76:6606.

The following properties are desirable in the design of an adenovirus vector to transfer a DNA molecule that encodes a biologically active, functional, truncated CFTR polypeptide to the airway cells of a CF patient, where the DNA can be expressed to provide a functional product that complements the CF defect. The vector should allow sufficient expression of truncated CFTR polypeptide, while producing minimal viral gene expression. The virus construct should also contain sufficient transcriptional and translational regulatory elements, as described above, so as to provide for sufficient production of functional truncated CFTR polypeptide that results in sufficient functional

regulated chloride ion channel activity in the membranes of treated cells in order to effect complementation of the CF defect. Moreover, it is to be expected that the expression of the smaller DNA molecules encoding truncated CFTR polypeptides are more efficiently expressed than DNA encoding full length CFTR, leading to higher levels of polypeptide product being produced. This increased expression would provide a greater likelihood of producing functional protein at the target cell membrane.

In addition, there should be minimal viral DNA replication and ideally no virus replication. Finally, recombination to produce new viral sequences and complementation to allow growth of the defective virus in the patient should be minimized.

A first generation adenovirus encoding full length CFTR has been prepared and includes viral DNA derived from the common, relatively benign, adenovirus 2 serotype. A similar vector can be prepared to express DNA molecules encoding truncated CFTR polypeptide according to the present invention. The Ela and Elb regions of the viral genome, which are involved in early stages of viral replication have been deleted. Their removal impairs viral gene expression and viral replication. The protein products of these genes also

have immortalizing and transforming function in some non-permissive cells.

A DNA molecule including a coding sequence for a truncated CFTR polypeptide can be inserted into the viral genome in place of the Ela/Elb region and transcription of the coding sequence will be driven by the endogenous Ela promoter. This is a moderately strong promoter that is functional in a variety of cells. In contrast to some adenovirus vectors (*see, e.g.*, Rosenfeld, M. et al. (1992) *Cell* 68:143), this adenovirus retains the E3 viral coding region. As a consequence of the inclusion of E3, the length of the adenovirus-CFTR DNA is greater than that of the wild-type adenovirus. The greater length of the recombinant viral DNA renders it more difficult to package. This means that the growth of the Ad2/CFTR virus is impaired even in permissive cells that provide the missing Ela and Elb functions.

The E3 region of the Ad2 vector encodes a variety of proteins. One of these proteins, gp 19, is believed to interact with and prevent presentation of class I proteins of the major histocompatibility complex (MHC). *See, e.g.*, Gooding, C.R. and Wold, W. S.M. (1990) *Crit. Rev. Immunol.* 10:53. This property prevents recognitcn of the infected cells and thus may allow viral latency. The presence of E3 sequences, therefore, has two useful attributes; first, the large size of the

viral DNA renders it doubly defective for replication (*i.e.*, it lacks early functions and is packaged poorly) and second, the absence of MHC presentation could be useful in later applications of Ad2/truncated CFTR in gene therapy involving multiple administrations, because it may avoid an immune response to recombinant virus-containing cells.

Not only are there advantages associated with the presence of E3; there may be disadvantages associated with its absence. Studies of E3 deleted virus in animals have suggested that they result in a more severe pathology. *See, e.g.*, Gingsberg, H.S. et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:3823. Furthermore, E3 deleted virus, such as might be obtained by recombination of an E1 plus E3 deleted virus with wild-type virus, is reported to outgrow wild-type in tissue culture. *See, e.g.*, Barkner, K.L. and Sharp, P. (1983) *Nucleic Acids Research* 11:6003. By contrast, however, a recent report of an E3 replacement vector encoding hepatitis B surface antigen, suggests that when delivered as a live enteric vaccine, such a virus replicates poorly in human compared to wild-type.

The adenovirus vector Ad2 vector can be grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express E1a and E1b, which

complement the defective adenoviruses by providing the products of the genes deleted from the vector.

In addition to Ad2, other varieties of adenovirus (*e.g.*, Ad3, Ad5, and Ad7) may also prove useful as gene therapy vectors. This may prove essential if immune response against a single serotype reduces the effectiveness of the therapy.

In addition, safer second-generation Ad vector systems are being produced. For example, an adenoviral construct expressing only the open reading frame 6 (ORF6) of adenoviral early region 4 (E4) from the E4 promoter and which is deleted for all other known E4 open reading frames has been constructed. *See, e.g.*, WO 94/12649. Expression of E4 ORF3 is also sufficient to provide E4 functions required for DNA replication and late protein synthesis. However, it provides these functions with reduced efficiency compared to expression of ORF6, which will likely result in lower levels of virus production. Therefore expressing ORF6, rather than ORF3, appears to be a better choice for producing recombinant adenovirus vectors.

The E4 region of adenovirus is suspected to have a role in viral DNA replication, late MRNA synthesis and host protein synthesis shut off, as well as in viral assembly. *See, e.g.*, Falgout, B. and G. Ketner (1987) *J Virol*. 61:3759-3768. Adenovirus early region 4 is

required for efficient virus particle assembly. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *See, e.g.*, Halbert, D.N. et al. (1985) *J. Virol.* 56:250-257.

The deletion of non-essential open reading frames of E4 increases the cloning capacity of recombinant adenovirus vectors by approximately 2 kb of insert DNA without significantly reducing the viability of the virus in cell culture. When placed in combination with deletions in the E1 and/or E3 regions of adenovirus vectors, the theoretical insert capacity of the resultant vectors is increased to 8-9 kb. An example of where this increased cloning capacity may prove useful is in the development of a gene therapy vector encoding CFTR, using the DNA molecules of the present invention. As described above, first generation adenoviral vectors approach the maximum packaging capacity for viral DNA encapsulation. As a result, this virus grows poorly and may occasionally give rise to defective progeny. Including an E4 deletion in the adenovirus vector should alleviate these problems. In addition, it allows flexibility in the choice of promoters to drive expression of DNA molecules encoding CFTR or truncated CFTR polypeptide as provided herein from the virus. For example, strong promoters such as the adenovirus major late promoter, the cytomegalovirus

immediate early promoter or a cellular promoter such as the CFTR promoter itself, which may be too large for first-generation adenovirus, can be used to drive expression.

In addition, as noted above, by expressing only ORF6 of E4, second generation Ad vectors should be safer for use in gene therapy. Although ORF6 expression is sufficient for viral DNA replication and late protein synthesis in immortalized cells, it has been suggested that ORF6/7 of E4 may also be required in non-dividing primary cells. *See, e.g.*, Hemstrom, C. et al. (1991) *J. Virol.* 65:1440-1449. The 19 kD protein produced from ORF6/7 complexes with and activates cellular transcription factor E2F, which is required for maximal activation of early region 2. Early region 2 encodes proteins required for viral DNA replication. Activated transcription factor E2F is present in proliferating cells and is involved in the expression of genes required for cell proliferation (*e.g.*, DHFR, c-myc), whereas activated E2F is present in lower levels in non-proliferating cells. Therefore, the expression of only ORF6 of E4 should allow the virus to replicate normally in tissue culture cells (*e.g.*, 293 cells), but the absence of ORF6/7 would prevent the potential activation of transcription factor E2F in non-dividing

primary cells and thereby reduce the potential for unwanted viral DNA replication.

The present invention is further illustrated by the following examples which in no way should be construed as being further limiting. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

EXAMPLE 1: EXPRESSION OF D836X IN HeLa CELLS

D836X, a DNA molecule including a nucleotide sequence coding for a truncated CFTR polypeptide, including the MSD-1, NBD-1 and R domains of wild-type human CFTR, was constructed in the vaccinia virus expression plasmid pTM-CFTR4 (Cheng et al., Cell 63, 827-834 1990), as previously described (Kunkel, Proc Natl Acad Sci. U.S.A. 82, 488-492 1985; Gregory et al., Nature 347, 382-386 1990). The nucleotide sequence of the D836X DNA molecule is set forth as SEQ ID NO. 1. This DNA molecule encodes a truncated CFTR polypeptide that includes the amino terminal portion of human CFTR, amino acids 1-836. This truncated CFTR polypeptide sequence, whose amino acid sequence is set forth as SEQ ID NO. 2, includes the MSD-1, NBD-1 and R domains (R

domain being defined as the boundaries of exon 13, residues 590 to 830 of CFTR). Thus, truncated CFTR polypeptide, as exemplified by the polypeptide product of DNA construct, D836X, is lacking the approximately 600+ amino acids at the carboxy terminus of humanCFTR, including the MSD-2 and NBD-2 domains.

The DNAs coding for wild-type human CFTR and the truncated CFTR polypeptide (D836X) were transiently expressed in HeLa cells using the vaccinia virus/bacteriophage T7 hybrid expression system, as previously described (Elroy-Stein et al. (1989), *Proc Natl Acad Sci U.S.A.* 86:6126-6130; Anderson et al. (1991), *Cell* 67:775-784). Cells (60-80% confluent, 8-24 h after seeding) were infected at a multiplicity of infection of 10-20. For protein expression studies, cell lysates were harvested 15 h after infection. For electrophysiology, cells were assayed for Cl^-channel function 15-30 h after infection.

CFTR and D836X polypeptide product were immuno-precipitated from digitonin-solubilized lysates of HeLa cells using two different monoclonal antibodies ("Ab"), the first, Ab M13-1, directed against the R domain (exon 13/β-galactosidase fusion protein) and the second, Ab M24-1, directed against the carboxyl terminus (amino acids 1477-1480) of CFTR (Gregory et al. (1990), *Nature* 347:382-386 1990; Marshall et al. (1995), *J. Biol Chem.*,

In Press). Antibody-polypeptide complexes were labeled by phosphorylation with [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase (PKA), electrophoresed on 8% SDS-polyacrylamide gels and autoradiographed (Gregory et al. (1990), *Nature* 347:382-386; Cheng et al. (1990), *Cell* 63:827-834).

Fig. 1 is an autoradiogram showing the results of these experiments. In the figure, CFTR (lanes 2 and 5) and D83X (lanes 3 and 6) were immunoprecipitated with anti-R domain antibody (M13-1) (lanes 1-3) or with anti-CFTR carboxy terminal antibody (M24-1). Lanes 1 and 4 are controls representing the results of immunoprecipitation of cell lysates transfected with the parent plasmid (pTM-1).

The results depicted in Fig. 1 show that M13-1 (lane 3), but not M24-1 (lane 6) precipitates a polypeptide of about 85 kd, consistent with the predicted size of the D836X polypeptide product (about 92 kd). The results further indicate that the D836X encoded polypeptide lacks the carboxy terminal portion of CFTR (including MSD-2 and NBD-2 domains).

EXAMPLE 2: ELECTROPHYSIOLOGY STUDIES

To assess the function of the D836X encoded polypeptide product compared with wild-type human CFTR, the patch clamp technique was used. Whole-cell and

single-channel currents were recorded, as previously described (Hamill et al. (1991), *Pfluegers Arch.* 391:85-100; Sheppard et al. (1993), *Nature* 362:160-164). Experiments were conducted at 34-36C°. The established sign convention was used throughout. Liquid junction potentials and potentials at the tip of the patch-pipette were measured and I-V relationships corrected for the corresponding offset.

For whole-cell experiments, the pipette (internal) solution contained (in mM): 120 N-methyl-D-glucamine (NMDG), 85 aspartic acid, 3 $MgCl_2$, 1 CsEGTA (ethyleneglycol-bis-(β-aminoethylether) N,N,N′,N′,-tetraacetic acid, cesium salt), 1 MGATP and 5 TES (N-Tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid), pH 7.3 with HCl ($[Cl^-]$, 43 mM; $[Ca^{2+}]$free $<10^{-8}$ M). The bath (external) solution contained (in mM): 140 NaCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 10 dextrose and 10 TES, pH 7.3 with NAOH ($[Cl^-]$, 142 mM). Whole-cell currents were filtered at 0.5 kHz and digitized at 2 kHz.

For experiments with excised inside-out membrane patches, the pipette (extracellular) solution contained (in mM): 140 NMDG, 140 aspartic acid, 5 $CaCl_2$, 2 $MgSO_4$ and 10 TES, pH 7.3 with Tris ($[Cl^-]$, 10 mM). The bath (intracellular) solution contained (in mM): 140 NMDG, 3 $MgCl_2$, 1 CsEGTA and 10 TES, pH 7.3 with HCl ($[Cl^-]$, 147 mM; $[Ca^{2+}]$free$<10^{-8}$ M). Single-channel

currents were filtered at 1 kHz and digitized at 10 kHz. Single-channel current amplitudes were determined from the fit of Gaussian distributions to current amplitude histograms. The fit of linear least squares regression lines to single-channel I-V relationships was used to determine single-channel conductance at negative voltages, where the I-V relationship was linear (Sheppard et al., Nature 362, 160-164 1993). Single-channel open-state probability (P_O) was measured in patches containing ≤ four (4) channels and in current recordings of at least 100 s duration. The number of channels in each patch was determined from the maximum number simultaneously open with 2.27 mM MGATP in the intracellular solution after PKA-dependent phosphorylation. Data were analyzed using pCIamp software (Axon Instruments, Inc., Foster City, CA).

EXAMPLE 3: ASSESSMENT OF FUNCTIONAL Cl^-CHANNEL ACTIVITY USING SPQ

cAMP-dependent Cl^-channel activity can be assessed using the halide-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ) (Molecular Probes), as previously described (Illsley and Verkman (1987) *Biochem.* 26:1215-1219). In this assay, an increase in halide permeability results in a more rapid increase in SPQ fluorescence. The rate of change rather than the absolute change in fluorescence is the important

variable in assessing anion permeability. Differences between groups in absolute levels may reflect quantitative differences between groups in SPQ loading, size of cells or number of cells studied (Illsley and Verkman, 1987). Fluorescence of SPQ in single cells can be measured with a Nikon inverted microscope, a digital imaging system from Universal Imaging and a Hamamatsu ICCD camera.

EXAMPLE 4: EXPRESSION OF D836X GENERATES cAMP-REGULATED Cl^- CURRENTS

To assess the function of the D836X polypeptide product compared with wild-type human CFTR, the patch-clamp technique of Example 2 was used. Table 1 set forth below, compares the properties of D836X whole-cell currents with those of wild-type CFTR. Under basal conditions, D836X whole-cell currents were small, but the amount of basal current appeared to be greater than that observed either in cells infected with control virus alone or in cells expressing wild-type CFTR. However, the difference was not statistically significant ($p = 0.23$). Addition of cAMP agonists reversibly activated whole-cell current in cells expressing D836X, although the magnitude of the increase was much less than that observed in cells expressing wild-type CFTR. D836X whole-cell currents, like those of wild-type CFTR, showed no evidence of voltage-dependent activation or

inactivation either before or after stimulation (not shown).

Under basal conditions, whole-cell currents from cells expressing the D836X construct had a relatively linear current-voltage (I-V) relationship and a reversal potential consistent with Cl^-selectivity, whereas basal whole-cell currents from cells expressing wild-type CFTR were much less Cl^-selective (Table 1). However, after addition of cAMP agonists, whole-cell currents from both groups of cells were Cl^-selective. The anion permeability and conductance sequences of D836X whole-cell currents were similar to those of wild-type CFTR ($Br^->Cl^->I^-$; Table 1).

Although many properties of the D836X whole-cell currents resembled those of wild-type human CFTR, two differences were observed. First, the tendency for cells expressing D836X to have larger basal whole-cell currents, and more convincingly, the Cl^-selectivity of basal currents suggested that D836X channels might have some activity even without cAMP stimulation (the pipette solution contained 1 mM MgATP). Second, the magnitude of cAMP-activated D836X whole-cell currents was reduced compared to that of wild-type CFTR ($p<0.001$).

These data suggested that the amino-terminal portion of CFTR, *i.e.*, a truncated CFTR polypeptide as

encoded by the D836X DNA construct, forms a regulated Cl^-channel. In contrast, the carboxyl-terminal portion of CFTR (R domain, MSD2 and NBD2, containing residues 1,2 and 708-1480) did not generate functional Cl^-channels despite producing protein of the expected size and immunoreactivity (not shown).

TABLE 1

Comparison of D836X and Wild-Type CFTR Whole-Cell Currents

Con-struct	Current at +50 mV (pA/pF)				Cell Capacitance
	n	Basal	cAMP	Wash	(pF)
D836X	10	4.5 ± 1.7	10.6 ± 3.0	2.7 ± 1.1	42.4 ± 3.5
CFTR	6	1.8 ± 0.2	99.0 ± 20.8	4.7 ± 0.9	35.6 ± 3.1

Con-struct	Anion-to-Cation Permeability (P_{Na}/P_{Cl})			Relative Anion Permeability (P_X/P_{Cl})			
	n	Basal	cAMP	n	Br^-	Cl^-	I^-
D836X	6	0.14 ± 0.03	0.17 ± 0.02	3-4	1.26 ± 0.03	1.0	0.65 ± 0.10
CFTR	6	0.48 ± 0.10	0.03 ± 0.02	3-4	1.26 ± 0.06	1.0	0.66 ± 0.08

Con-struct	Relative Anion Conductance (G_X/G_{Cl})			
	n	Br^-	Cl^-	I^-
D836X	3-4	1.00 ± 0.19	1.0	0.33 ± 0.06
CFTR	3-4	1.21 ± 0.12	1.0	0.17 ± 0.01

Data are mean ± SEM of values calculated from currents in individual cells (n, number of cells) under basal, cAMP (10μM forskolin, 100μM IBMX, and 500μM 8-(4-chlorophenylthio)-adenosine 3'-5'-cyclic monophosphate sodium salt, CPT-cAMP) and wash conditions. (Currents at +50 mV for cells infected with control virus alone were 1.3 ± 0.3 pA/pF (basal) and 0.3 ± 0.0 pA/pF (cAMP); cell capacitance was 31.6 ± 5.7; n = 6. Permeability and conductance ratios of anions (X^-) were determined in the presence of cAMP agonists, as previously described (Anderson et al., 1991b). I-V relationships were obtained by a 400 ms ramp of voltage; holding voltage was -40 mV.

EXAMPLE 5: COMPARISON OF THE SINGLE Cl^- CHANNEL PROPERTIES OF THE D836X POLYPEPTIDE PRODUCT AND WILD-TYPE CFTR

To better understand the function of the truncated CFTR polypeptides of the invention, including the amino-terminal portion of CFTR, the single-channel properties of the D836X polypeptide product (SEQ ID NO. 2) were compared with those of wild-type human CFTR, using excised inside-out membrane patches as provided in Example 2. After excision of membrane patches from cells expressing D836X, no channel activity was observed. However, as shown in Fig. 2, which provides a comparison of D836X polypeptide product and wild-type CFTR Cl^- channel activity before and after PKA-dependent phosphorylation, as soon as ATP was added to the cytosolic surface (0.88 mM MGATP), channels opened and the open-state probability (P_o) was approximately 0.1.

In Fig. 2A, representative single-channel recordings from excised inside-out membrane patches (as provided in Example 2) from HeLa cells transiently expressing either D836X or wild-type CFTR are shown. ATP (0.88 mM MgATP) and the catalytic subunit of PKA (75 nM) were added to the intracellular solution, as indicated. Voltage was -91 mV (D836X and -88 mV (CFTR), respectively. Dashed lines indicate the closed channel state and downward deflections correspond to channel openings.

In Fig. 2(B), P_o values before and after PKA-dependent phosphorylation are shown. Solid symbols connected by lines represent individual experiments while open symbols shown mean ± SEM values; n = 6. Voltage was -90 or -30 mV; P_o is voltage independent (D836X P_o at -90 and -30 mV was 0.39 ± 0.11 and 0.30 ± 0.10, respectively, after PKA addition, n = 3, p = 0.19; CFTR, see Sheppard et al., 1993, Nature 362:160-164).

The result obtained with D836X contrasts sharply with the behavior of wild-type human CFTR: in the presence of intracellular MgATP alone, the P_o of wild-type CFTR Cl^- channels was zero (Fig. 2B). Phosphorylation with PKA (75 riM) significantly increased the P_o of D836X channels. As previously described, phosphorylation with PKA was required to open wild-type CFTR Cl^-channels (Chang et al. (1993), *J. Biol. Chem.* 268:11304-11311; Cheng et al. (1991), *Cell* 66:1027-1036; Rich et al. (1993) *J. Biol. Chem.* 268:20259-20267). Once phosphorylated, D836X and CFTR channels had similar P_o values (Fig. 2B). These data suggest that some aspects of the relationship between the R domain and the rest of the channel may be altered in the D836X polypeptide product, while others appear to remain intact.

The conductive properties of the D836X polypeptide product appeared to be the same as those of wild-type CFTR. Fig. 2A shows that the single-channel

current amplitude of D836X was similar to CFTR. In addition, the single-channel slope conductance of D836X (8.03 ± 0.23 pS; n = 6) was not different from that of wild-type CFTR (8.29 ± 0.15 pS; n = 4).

Whole-cell current (I) is determined by the number of channels in the plasma membrane (N), Po, and single-channel current amplitude (i): $I = N \cdot i \cdot P_o$. The finding that the D836X polypeptide product has i and P_o values comparable to those of wild-type CFTR suggests that whole-cell current is reduced in cells expressing D836X most likely because N, the number of functional Cl^-channels in the plasma membrane, is decreased. Consistent with this interpretation is the finding that cAMP-activated whole-cell currents were observed in 10 of 59 cells expressing D836X (17%), compared with 10 of 14 cells expressing wild-type CFTR (71%). Thus, D836X was not as efficient as wild-type human CFTR at generating functional channels.

Approaches to increasing the efficiency of D836X and other truncated CFTR polypeptide molecules in generating functional channels are discussed *infra* (Example 8).

EXAMPLE 6: REGULATION OF D836X Cl^-CHANNELS BY INTRACELLULAR NUCLEOTIDES

Once phosphorylated with PKA, wild-type human CFTR Cl^-channels require cytosolic MgATP to open. Previous studies have suggested that MgATP interacts with both NBD-1 and NBD-2 to regulate the channel (Anderson and Welsh (1992), *Science* 257:1701-1704; Smit et al. (1993), *Proc. Natl. Acad. Sci. USA* 90:9963-9967). Because D836X lacks the NBD-2 domain, the interaction with intracellular nucleotides might be altered. To test this, the effect of intracellular MgATP on channels that had been phosphorylated with PKA was examined. As previously observed for wild-type CFTR, increasing concentrations of MgATP increased the activity of the D836X polypeptide product. This is shown in Fig. 3A and B.

Fig. 3A provides a comparison of the effect of different intracellular MgATP concentrations on the activity of single D836X and wild-type Cl^-channels. Voltage was -88 mV (D836X) and -83 mV (CFTR), respectively.

Fig. 3B shows the relationship between P_o and intracellular MgATP concentration for D836X (closed circle) and wild-type CFTR (closed square) Cl^-channels. Data points are mean ± SEM of n = 4-8 at each MgATP concentration. Voltage was -88 ± 1 mV (D836X) and -86 ± 1 mV (CFTR).

The results show, however, that D836X Cl^-channels had higher P_o values at MGATP concentrations between 0.09 and 0.88 mM than did wild-type CFTR Cl^-channels. This difference was particularly dramatic at the lowest MgATP concentrations tested. As was previously reported for wild-type CFTR (Anderson and Welsh (1992), *Science* 257:1701-1704), an Eadie-Hofstee plot of the D836X data generated a curved line (Fig. 3C is an Eadie-Hofstee plot of data shown in Fig. 3B). One possible interpretation of a curved line in such a plot is that there is kinetic cooperativity, perhaps with ATP interacting with more than one site in a functional channel.

Previous studies of wild-type human CFTR showed that cytosolic ADP inhibited the channel (Anderson and Welsh (1992), *Science* 257:1701-1704). Based on studies of variants containing site-directed mutations in the two NBDs, it was suggested that ADP interacted with NBD2. Therefore, whether ADP would inhibit D836X Cl^-channels, which lack an NBD-2 domain was of interest. Fig. 4A and B shows that intracellular ADP (1 mM) produced equivalent reductions in the activity of both D836X and wild-type channels.

Fig. 4A shows a comparison of the effect of ADP on the activity of three D836X Cl^-channels and a single wild-type Cl^-channel following PKA-dependent

phosphorylation. Note the change in scale. ATP (0.88 mM MgATP) and ADP (1 mM) were added to the intracellular solution as indicated. Voltage was -86 mV (D836X) and -83 mV (CFTR), respectively.

Fig. 4B shows the effect of 1 mM ADP on P_o values are mean ± SEM; n = 3. Voltage was -86 ± 1 mV (D836X) and -85 ± 2 mV (CFTR). Other details are as in Fig. 4A.

EXAMPLE 7: THE D836X POLYPEPTIDE PRODUCT SEDIMENTS AS A MULTIMER ON SUCROSE GRADIENT CENTRIFUGATION

Based on the results set forth in the previous Examples, it was asked whether the D836X polypeptide product exists as a multimer. To test this possibility, the migration of the D836 polypeptide on sucrose density gradients was examined.

Sucrose gradient centrifugation was carried out on Triton X-100 solubilized lysates from unlabeled cells (as in Example 1), centrifuged on 5% to 20% sucrose gradients (Gradient Master, BioComp Instruments Incorporated, Fredericton, Canada) in 0.5 M NaCl, 50 mM Tris-HCI, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, and 0.75 mM benzamidine-HCl with 0.1% TX-100 for 2.5 hr at 215,000 x g at 4°C. Gradients were fractionated from the top and fractions were immunoprecipitated with antibody M13-1 to the R domain, phosphorylated and

electrophoresed, as described in Example 1. The amount of immunoprecipitated and phosphorylated D836X or CFTR in each fraction was then quantitated by radioanalytic scanning (AMBIS Systems Inc., San Diego, California).

The results of sucrose gradient experiments are set forth in Fig. 5.

Fig. 5A and B are autoradiograms of D836X(A) and CFTR(B) after sedimentation on 5%-20% sucrose gradients, immunoprecipitation with M13-1, phosphorylation, and electrophoresis on SDS-PAGE. D836X and CFTR immunoprecipitated and phosphorylated from total lysate prior to centrifugation are on the right of each panel.

Fig. 5C shows the quantitative distribution of D836X (closed circle) and CFTR (closed square) in these sucrose gradients, measured as described in Experimental Procedures. Standard protein markers (bovine serum albumin, 67 kd; aldolase, 158 kd; catalase, 240 kd and thyroglobulin, 660 kd) were run in parallel gradients and their sedimentation is indicated.

It was found that in the presence of Triton X-100(TX-100), the D836X polypeptide sediments in the middle of a 5-20% sucrose gradient. This corresponds to the sedimentation of the standard protein marker aldolase (158 kDa), which is double that expected for the predicted molecular weight of a monomeric D836X

polypeptide product and is similar to the migration of wild-type CFTR. The D836X polypeptide product was found to also migrate in the same fractions as CFTR when sedimented in 1030% TX-100 gradients or in 5-20 or 10-30% 3-[(3-Cholamidopropyl)dimethyl-animonio]-1--propanesulfonate (CHAPS) gradients. These sedimentation patterns suggest that the D836X polypeptide product self-associates into a dimeric form that may be more stable than the single polypeptide.

EXAMPLE 8: WAYS TO INCREASE FUNCTIONAL D836X POLYPEPTIDE PRODUCTS AT THE CELL SURFACE MEMBRANE

It is believed that while D836X (and other DNA molecules coding for truncated CFTR polypeptide having regulated chloride ion channel activity) is efficiently expressed by cells containing the encoding DNA molecule, the above differences in Cl^-channel activity with wild-type CFTR result from decreased delivery of the expressed polypeptide product to the membrane where it acts as a functional Cl^-channel. There are several approaches that may be useful in increasing the delivery of the truncated CFTR polypeptide to the membrane to act as a functional regulated anion channel.

The first approach, discussed above, is to increase expression of a DNA molecule encoding a truncated CFTR polypeptide in order to maximize the

potential for synthesized polypeptide product to be translocated to the host cell membrane.

A second approach involves altering a DNA molecule encoding a truncated CFTR polypeptide (*e.g.*, D836X), so that the DNA would also encode a truncated CFTR polypeptide that includes a portion of the carboxy terminal tail of CFTR. Alternatively, the inclusion in the encoded polypeptide of certain portions of the CFTR transmembrane sequences (*e.g.*, the seventh and eighth transmembrane sequences that are found in MSD-2 of CFTR situated in the protein sequence right after the R domain) may increase the stability of the truncated CFTR ploypeptide and facilitate higher levels of functional Cl^-channels. Also, some combination of carboxy terminal tail and the seventh and eighth transmembrane sequences may be utilized. Evidence that this approach is feasible may be found in studies showing that some CFTR mutant proteins lacking domains are processed and delivered to the plasma membrane. *See*, *e.g.*, Rich et al. (1993), *Receipt. Chan.* 1:221-232.

A third approach is premised on the finding, as provided in Example 7, that the D836X polypeptide product appears to function as a dimer. One way of improving post-translation processing of truncated CFTR polypeptide (*e.g.*, D836X) is to stabilize the dimeric state of the protein. For example, the DNA coding for truncated CFTR

polypeptide may be modified (*e.g.*, by site-specific mutagenesis) so that the resulting polypeptide product includes cysteines that would form disulfide bridges, thereby stabilizing the dimer.

The dimerization might also be improved by including regions in the amino terminal tail, which could improve association of the subunits. For example, in the Drosophila Shaker B potassium channel, the amino-terminal tail helps the channel form a multimer. *See, e.g.*, Li et al. (1992), *Science* 257:1225-1230. Inclusion of such a tail or a related motif in the amino terminal tail or in the carboxy terminal tail of a truncated CFTR polypeptide of the invention might increase the association of subunits, thereby forming a more stable channel and thereby improving processing and delivery to the plasma membrane.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Sheppard, David N.

(ii) TITLE OF INVENTION: NOVEL GENES AND PROTEINS FOR TREATING CYSTIC FIBROSIS

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US S/N 08/216,971
(B) FILING DATE: 23-MAR-1994
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2640 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 133..2640

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AATTGGAAGC AAATGACATC ACAGCAGGTC AGAGAAAAAG GGTTGAGCGG CAGGCACCCA      60

GAGTAGTAGG TCTTTGGCAT TAGGAGCTTG AGCCCAGACG GCCCTAGCAG GGACCCCAGC     120

GCCCGAGAGA CC ATG CAG AGG TCG CCT CTG GAA AAG GCC AGC GTT GTC          168
              Met Gln Arg Ser Pro Leu Glu Lys Ala Ser Val Val
                1               5                  10

TCC AAA CTT TTT TTC AGC TGG ACC AGA CCA ATT TTG AGG AAA GGA TAC       216
Ser Lys Leu Phe Phe Ser Trp Thr Arg Pro Ile Leu Arg Lys Gly Tyr
         15                  20                  25

AGA CAG CGC CTG GAA TTG TCA GAC ATA TAC CAA ATC CCT TCT GTT GAT       264
Arg Gln Arg Leu Glu Leu Ser Asp Ile Tyr Gln Ile Pro Ser Val Asp
     30                  35                  40

TCT GCT GAC AAT CTA TCT GAA AAA TTG GAA AGA GAA TGG GAT AGA GAG       312
Ser Ala Asp Asn Leu Ser Glu Lys Leu Glu Arg Glu Trp Asp Arg Glu
 45                  50                  55                  60

CTG GCT TCA AAG AAA AAT CCT AAA CTC ATT AAT GCC CTT CGG CGA TGT       360
Leu Ala Ser Lys Lys Asn Pro Lys Leu Ile Asn Ala Leu Arg Arg Cys
                 65                  70                  75

TTT TTC TGG AGA TTT ATG TTC TAT GGA ATC TTT TTA TAT TTA GGG GAA       408
Phe Phe Trp Arg Phe Met Phe Tyr Gly Ile Phe Leu Tyr Leu Gly Glu
             80                  85                  90

GTC ACC AAA GCA GTA CAG CCT CTC TTA CTG GGA AGA ATC ATA GCT TCC       456
Val Thr Lys Ala Val Gln Pro Leu Leu Leu Gly Arg Ile Ile Ala Ser
         95                  100                 105

TAT GAC CCG GAT AAC AAG GAG GAA CGC TCT ATC GCG ATT TAT CTA GGC       504
Tyr Asp Pro Asp Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly
    110                 115                 120

ATA GGC TTA TGC CTT CTC TTT ATT GTG AGG ACA CTG CTC CTA CAC CCA       552
Ile Gly Leu Cys Leu Leu Phe Ile Val Arg Thr Leu Leu Leu His Pro
125                 130                 135                 140

GCC ATT TTT GGC CTT CAT CAC ATT GGA ATG CAG ATG AGA ATA GCT ATG       600
Ala Ile Phe Gly Leu His His Ile Gly Met Gln Met Arg Ile Ala Met
                145                 150                 155

TTT AGT TTG ATT TAT AAG AAG ACT TTA AAG CTG TCA AGC CGT GTT CTA       648
Phe Ser Leu Ile Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu
            160                 165                 170

GAT AAA ATA AGT ATT GGA CAA CTT GTT AGT CTC CTT TCC AAC AAC CTG       696
Asp Lys Ile Ser Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu
        175                 180                 185

AAC AAA TTT GAT GAA GGA CTT GCA TTG GCA CAT TTC GTG TGG ATC GCT       744
Asn Lys Phe Asp Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala
    190                 195                 200

CCT TTG CAA GTG GCA CTC CTC ATG GGG CTA ATC TGG GAG TTG TTA CAG       792
Pro Leu Gln Val Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln
205                 210                 215                 220
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GCG TCT GCC TTC TGT GGA CTT GGT TTC CTG ATA GTC CTT GCC CTT TTT                840
Ala Ser Ala Phe Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe
                225                 230                 235

CAG GCT GGG CTA GGG AGA ATG ATG ATG AAG TAC AGA GAT CAG AGA GCT                888
Gln Ala Gly Leu Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala
            240                 245                 250

GGG AAG ATC AGT GAA AGA CTT GTG ATT ACC TCA GAA ATG ATT GAA AAT                936
Gly Lys Ile Ser Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn
        255                 260                 265

ATC CAA TCT GTT AAG GCA TAC TGC TGG GAA GAA GCA ATG GAA AAA ATG                984
Ile Gln Ser Val Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met
    270                 275                 280

ATT GAA AAC TTA AGA CAA ACA GAA CTG AAA CTG ACT CGG AAG GCA GCC               1032
Ile Glu Asn Leu Arg Gln Thr Glu Leu Lys Leu Thr Arg Lys Ala Ala
285                 290                 295                 300

TAT GTG AGA TAC TTC AAT AGC TCA GAA TTC TTC TTC TCA GGG TTC TTT               1080
Tyr Val Arg Tyr Phe Asn Ser Ser Glu Phe Phe Phe Ser Gly Phe Phe
                305                 310                 315

GTG GTG TTT TTA TCT GTG CTT CCC TAT GCA CTA ATC AAA GGA ATC ATC               1128
Val Val Phe Leu Ser Val Leu Pro Tyr Ala Leu Ile Lys Gly Ile Ile
            320                 325                 330

CTC CGG AAA ATA TTC ACC ACC ATC TCA TTC TGC ATT GTT CTG CGC ATG               1176
Leu Arg Lys Ile Phe Thr Thr Ile Ser Phe Cys Ile Val Leu Arg Met
        335                 340                 345

GCG GTC ACT CGG CAA TTT CCC TGG GCT GTA CAA ACA TGG TAT GAC TCT               1224
Ala Val Thr Arg Gln Phe Pro Trp Ala Val Gln Thr Trp Tyr Asp Ser
    350                 355                 360

CTT GGA GCA ATA AAC AAA ATA CAG GAT TTC TTA CAA AAG CAA GAA TAT               1272
Leu Gly Ala Ile Asn Lys Ile Gln Asp Phe Leu Gln Lys Gln Glu Tyr
365                 370                 375                 380

AAG ACA TTG GAA TAT AAC TTA ACG ACT ACA GAA GTA GTG ATG GAG AAT               1320
Lys Thr Leu Glu Tyr Asn Leu Thr Thr Thr Glu Val Val Met Glu Asn
                385                 390                 395

GTA ACA GCC TTC TGG GAG GAG GGA TTT GGG GAA TTA TTT GAG AAA GCA               1368
Val Thr Ala Phe Trp Glu Glu Gly Phe Gly Glu Leu Phe Glu Lys Ala
            400                 405                 410

AAA CAA AAC AAT AAC AAT AGA AAA ACT TCT AAT GGT GAT GAC AGC CTC               1416
Lys Gln Asn Asn Asn Asn Arg Lys Thr Ser Asn Gly Asp Asp Ser Leu
        415                 420                 425

TTC TTC AGT AAT TTC TCA CTT CTT GGT ACT CCT GTC CTG AAA GAT ATT               1464
Phe Phe Ser Asn Phe Ser Leu Leu Gly Thr Pro Val Leu Lys Asp Ile
    430                 435                 440

AAT TTC AAG ATA GAA AGA GGA CAG TTG TTG GCG GTT GCT GGA TCC ACT               1512
Asn Phe Lys Ile Glu Arg Gly Gln Leu Leu Ala Val Ala Gly Ser Thr
445                 450                 455                 460
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GGA GCA GGC AAG ACT TCA CTT CTA ATG ATG ATT ATG GGA GAA CTG GAG      1560
Gly Ala Gly Lys Thr Ser Leu Leu Met Met Ile Met Gly Glu Leu Glu
                465                 470                 475

CCT TCA GAG GGT AAA ATT AAG CAC AGT GGA AGA ATT TCA TTC TGT TCT      1608
Pro Ser Glu Gly Lys Ile Lys His Ser Gly Arg Ile Ser Phe Cys Ser
            480                 485                 490

CAG TTT TCC TGG ATT ATG CCT GGC ACC ATT AAA GAA AAT ATC ATC TTT      1656
Gln Phe Ser Trp Ile Met Pro Gly Thr Ile Lys Glu Asn Ile Ile Phe
        495                 500                 505

GGT GTT TCC TAT GAT GAA TAT AGA TAC AGA AGC GTC ATC AAA GCA TGC      1704
Gly Val Ser Tyr Asp Glu Tyr Arg Tyr Arg Ser Val Ile Lys Ala Cys
    510                 515                 520

CAA CTA GAA GAG GAC ATC TCC AAG TTT GCA GAG AAA GAC AAT ATA GTT      1752
Gln Leu Glu Glu Asp Ile Ser Lys Phe Ala Glu Lys Asp Asn Ile Val
525                 530                 535                 540

CTT GGA GAA GGT GGA ATC ACA CTG AGT GGA GGT CAA CGA GCA AGA ATT      1800
Leu Gly Glu Gly Gly Ile Thr Leu Ser Gly Gly Gln Arg Ala Arg Ile
                545                 550                 555

TCT TTA GCA AGA GCA GTA TAC AAA GAT GCT GAT TTG TAT TTA TTA GAC      1848
Ser Leu Ala Arg Ala Val Tyr Lys Asp Ala Asp Leu Tyr Leu Leu Asp
            560                 565                 570

TCT CCT TTT GGA TAC CTA GAT GTT TTA ACA GAA AAA GAA ATA TTT GAA      1896
Ser Pro Phe Gly Tyr Leu Asp Val Leu Thr Glu Lys Glu Ile Phe Glu
        575                 580                 585

AGC TGT GTC TGT AAA CTG ATG GCT AAC AAA ACT AGG ATT TTG GTC ACT      1944
Ser Cys Val Cys Lys Leu Met Ala Asn Lys Thr Arg Ile Leu Val Thr
    590                 595                 600

TCT AAA ATG GAA CAT TTA AAG AAA GCT GAC AAA ATA TTA ATT TTG CAT      1992
Ser Lys Met Glu His Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu His
605                 610                 615                 620

GAA GGT AGC AGC TAT TTT TAT GGG ACA TTT TCA GAA CTC CAA AAT CTA      2040
Glu Gly Ser Ser Tyr Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu
                625                 630                 635

CAG CCA GAC TTT AGC TCA AAA CTC ATG GGA TGT GAT TCT TTC GAC CAA      2088
Gln Pro Asp Phe Ser Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln
            640                 645                 650

TTT AGT GCA GAA AGA AGA AAT TCA ATC CTA ACT GAG ACC TTA CAC CGT      2136
Phe Ser Ala Glu Arg Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg
        655                 660                 665

TTC TCA TTA GAA GGA GAT GCT CCT GTC TCC TGG ACA GAA ACA AAA AAA      2184
Phe Ser Leu Glu Gly Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys
    670                 675                 680

CAA TCT TTT AAA CAG ACT GGA GAG TTT GGG GAA AAA AGG AAG AAT TCT      2232
Gln Ser Phe Lys Gln Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser
685                 690                 695                 700
```

```
ATT CTC AAT CCA ATC AAC TCT ATA CGA AAA TTT TCC ATT GTG CAA AAG      2280
Ile Leu Asn Pro Ile Asn Ser Ile Arg Lys Phe Ser Ile Val Gln Lys
                705                 710                 715

ACT CCC TTA CAA ATG AAT GGC ATC GAA GAG GAT TCT GAT GAG CCT TTA      2328
Thr Pro Leu Gln Met Asn Gly Ile Glu Glu Asp Ser Asp Glu Pro Leu
            720                 725                 730

GAG AGA AGG CTG TCC TTA GTA CCA GAT TCT GAG CAG GGA GAG GCG ATA      2376
Glu Arg Arg Leu Ser Leu Val Pro Asp Ser Glu Gln Gly Glu Ala Ile
        735                 740                 745

CTG CCT CGC ATC AGC GTG ATC AGC ACT GGC CCC ACG CTT CAG GCA CGA      2424
Leu Pro Arg Ile Ser Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg
    750                 755                 760

AGG AGG CAG TCT GTC CTG AAC CTG ATG ACA CAC TCA GTT AAC CAA GGT      2472
Arg Arg Gln Ser Val Leu Asn Leu Met Thr His Ser Val Asn Gln Gly
765                 770                 775                 780

CAG AAC ATT CAC CGA AAG ACA ACA GCA TCC ACA CGA AAA GTG TCA CTG      2520
Gln Asn Ile His Arg Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu
                785                 790                 795

GCC CCT CAG GCA AAC TTG ACT GAA CTG GAT ATA TAT TCA AGA AGG TTA      2568
Ala Pro Gln Ala Asn Leu Thr Glu Leu Asp Ile Tyr Ser Arg Arg Leu
            800                 805                 810

TCT CAA GAA ACT GGC TTG GAA ATA AGT GAA GAA ATT AAC GAA GAA GAC      2616
Ser Gln Glu Thr Gly Leu Glu Ile Ser Glu Glu Ile Asn Glu Glu Asp
        815                 820                 825

TTA AAG GAG TGC CTT TTT GAT GAT                                     2640
Leu Lys Glu Cys Leu Phe Asp Asp
    830                 835
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 836 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Gln Arg Ser Pro Leu Glu Lys Ala Ser Val Val Ser Lys Leu Phe
  1               5                  10                  15

Phe Ser Trp Thr Arg Pro Ile Leu Arg Lys Gly Tyr Arg Gln Arg Leu
            20                  25                  30

Glu Leu Ser Asp Ile Tyr Gln Ile Pro Ser Val Asp Ser Ala Asp Asn
        35                  40                  45

Leu Ser Glu Lys Leu Glu Arg Glu Trp Asp Arg Glu Leu Ala Ser Lys
    50                  55                  60

Lys Asn Pro Lys Leu Ile Asn Ala Leu Arg Arg Cys Phe Phe Trp Arg
65                  70                  75                  80
```

```
Phe Met Phe Tyr Gly Ile Phe Leu Tyr Leu Gly Glu Val Thr Lys Ala
                85                  90                  95

Val Gln Pro Leu Leu Leu Gly Arg Ile Ile Ala Ser Tyr Asp Pro Asp
            100                 105                 110

Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly Ile Gly Leu Cys
        115                 120                 125

Leu Leu Phe Ile Val Arg Thr Leu Leu Leu His Pro Ala Ile Phe Gly
    130                 135                 140

Leu His His Ile Gly Met Gln Met Arg Ile Ala Met Phe Ser Leu Ile
145                 150                 155                 160

Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu Asp Lys Ile Ser
                165                 170                 175

Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu Asn Lys Phe Asp
            180                 185                 190

Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala Pro Leu Gln Val
        195                 200                 205

Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln Ala Ser Ala Phe
    210                 215                 220

Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe Gln Ala Gly Leu
225                 230                 235                 240

Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala Gly Lys Ile Ser
                245                 250                 255

Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn Ile Gln Ser Val
            260                 265                 270

Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met Ile Glu Asn Leu
        275                 280                 285

Arg Gln Thr Glu Leu Lys Leu Thr Arg Lys Ala Ala Tyr Val Arg Tyr
    290                 295                 300

Phe Asn Ser Ser Glu Phe Phe Phe Ser Gly Phe Phe Val Val Phe Leu
305                 310                 315                 320

Ser Val Leu Pro Tyr Ala Leu Ile Lys Gly Ile Ile Leu Arg Lys Ile
                325                 330                 335

Phe Thr Thr Ile Ser Phe Cys Ile Val Leu Arg Met Ala Val Thr Arg
            340                 345                 350

Gln Phe Pro Trp Ala Val Gln Thr Trp Tyr Asp Ser Leu Gly Ala Ile
        355                 360                 365

Asn Lys Ile Gln Asp Phe Leu Gln Lys Gln Glu Tyr Lys Thr Leu Glu
    370                 375                 380

Tyr Asn Leu Thr Thr Thr Glu Val Val Met Glu Asn Val Thr Ala Phe
385                 390                 395                 400

Trp Glu Glu Gly Phe Gly Glu Leu Phe Glu Lys Ala Lys Gln Asn Asn
                405                 410                 415
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Asn Asn Arg Lys Thr Ser Asn Gly Asp Asp Ser Leu Phe Phe Ser Asn
420 425 430

Phe Ser Leu Leu Gly Thr Pro Val Leu Lys Asp Ile Asn Phe Lys Ile
435 440 445

Glu Arg Gly Gln Leu Leu Ala Val Ala Gly Ser Thr Gly Ala Gly Lys
450 455 460

Thr Ser Leu Leu Met Met Ile Met Gly Glu Leu Glu Pro Ser Glu Gly
465 470 475 480

Lys Ile Lys His Ser Gly Arg Ile Ser Phe Cys Ser Gln Phe Ser Trp
485 490 495

Ile Met Pro Gly Thr Ile Lys Glu Asn Ile Ile Phe Gly Val Ser Tyr
500 505 510

Asp Glu Tyr Arg Tyr Arg Ser Val Ile Lys Ala Cys Gln Leu Glu Glu
515 520 525

Asp Ile Ser Lys Phe Ala Glu Lys Asp Asn Ile Val Leu Gly Glu Gly
530 535 540

Gly Ile Thr Leu Ser Gly Gly Gln Arg Ala Arg Ile Ser Leu Ala Arg
545 550 555 560

Ala Val Tyr Lys Asp Ala Asp Leu Tyr Leu Leu Asp Ser Pro Phe Gly
565 570 575

Tyr Leu Asp Val Leu Thr Glu Lys Glu Ile Phe Glu Ser Cys Val Cys
580 585 590

Lys Leu Met Ala Asn Lys Thr Arg Ile Leu Val Thr Ser Lys Met Glu
595 600 605

His Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu His Glu Gly Ser Ser
610 615 620

Tyr Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu Gln Pro Asp Phe
625 630 635 640

Ser Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln Phe Ser Ala Glu
645 650 655

Arg Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg Phe Ser Leu Glu
660 665 670

Gly Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys Gln Ser Phe Lys
675 680 685

Gln Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser Ile Leu Asn Pro
690 695 700

Ile Asn Ser Ile Arg Lys Phe Ser Ile Val Gln Lys Thr Pro Leu Gln
705 710 715 720

Met Asn Gly Ile Glu Glu Asp Ser Asp Glu Pro Leu Glu Arg Arg Leu
725 730 735

Ser Leu Val Pro Asp Ser Glu Gln Gly Glu Ala Ile Leu Pro Arg Ile
740 745 750

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Ser Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg Arg Arg Gln Ser
        755                 760                 765

Val Leu Asn Leu Met Thr His Ser Val Asn Gln Gly Gln Asn Ile His
    770                 775                 780

Arg Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu Ala Pro Gln Ala
785                 790                 795                 800

Asn Leu Thr Glu Leu Asp Ile Tyr Ser Arg Arg Leu Ser Gln Glu Thr
                805                 810                 815

Gly Leu Glu Ile Ser Glu Glu Ile Asn Glu Glu Asp Leu Lys Glu Cys
            820                 825                 830

Leu Phe Asp Asp
        835
```

CLAIMS

1. A DNA molecule that includes a nucleotide sequence encoding a truncated CFTR polypeptide that lacks a significant portion of the carboxy terminal amino acid sequence of wild-type human CFTR, wherein the truncated CFTR polypeptide possesses a biological activity significantly duplicative of wild-type human CFTR so as to function as a regulated epithelial cell anion channel.

2. A DNA molecule of Claim 1, wherein the encoded truncated CFTR polypeptide includes at least an MSD-1, an NBD-1 and R domain of human CFTR.

3. A DNA molecule of Claim 2, wherein the encoded truncated CFTR polypeptide provides a functional chloride ion channel and a regulator of the opening and closing of the chloride ion channel.

4. A DNA molecule of Claim 1, wherein the DNA molecule comprises a D836X construct which encodes the amino terminal 836 amino acids of human CFTR.

5. A DNA molecule having the nucleotide sequence set forth in SEQ ID NO. 1.

6. A host cell containing therein a DNA molecule according to any one of Claims 1-5.

7. A vector or vehicle for gene therapy containing a DNA molecule according to any one of Claims 1-5.

8. A vector according to Claim 7, wherein the vector is a virus.

9. A vector according to Claim 8, wherein the virus is selected from the group consisting of adenoviruses, adeno-associated viruses and retroviruses.

10. A vehicle according to Claim 7, wherein the vehicle is a lipid.

11. A host cell according to Claim 6, wherein the cell is an epithelial cell.

12. A pharmaceutical composition for gene therapy comprising the vector or vehicle of Claim 7 and a pharmaceutically acceptable carrier.

13. A truncated CFTR polypeptide that lacks a significant portion of the carboxy terminal amino acid

sequence of wild-type human CFTR, wherein the truncated CFTR polypeptide possesses a biological activity sufficiently duplicative of wild-type human CFTR so as to function as a regulated epithelial cell anion channel.

14. A truncated CFTR polypeptide of Claim 13, wherein the polypeptide includes at least an MSD-1, an NBD-1 and an R domain of human CFTR.

15. A truncated CFTR polypeptide of Claim 14 wherein the polypeptide provides a functional chloride ion channel and a regulator of the opening and closing of the chloride ion channel.

16. A truncated CFTR polypeptide including the amino terminal 836 amino acids of human CFTR.

17. A truncated CFTR polypeptide having the amino acid sequence set forth as SEQ ID NO. 2.

18. A truncated CFTR polypeptide encoded by a DNA molecule according to Claims 1-5.

19. A pharmaceutical composition for protein therapy of Cystic Fibrosis comprising a truncated CFTR

polypeptide according to any one of Claims 13-18 and a pharmaceutically acceptable carrier.

20. A method for treating Cystic Fibrosis in a patient comprising introducing into target cells in the patient a DNA molecule according to any one of Claims 105, wherein the introduction of the DNA molecule results in expression of the DNA molecule, such that a truncated CFTR polypeptide is produced, having a biological activity sufficiently duplicative of wild-type human CFTR so as to function as a regulated epithelial cell anion channel that complements the Cystic Fibrosis defect in the patient's cells.

21. A method according to Claim 20, wherein the target cells are epithelial cells of the patient's airways and lungs.

22. A method of treating Cystic Fibrosis in a patient comprising administering to the cells of the patient a truncated CFTR polypeptide of Claims 13-18 in an amount sufficient to provide the patient cells with functional regulated epithelial cell anion channel activity.

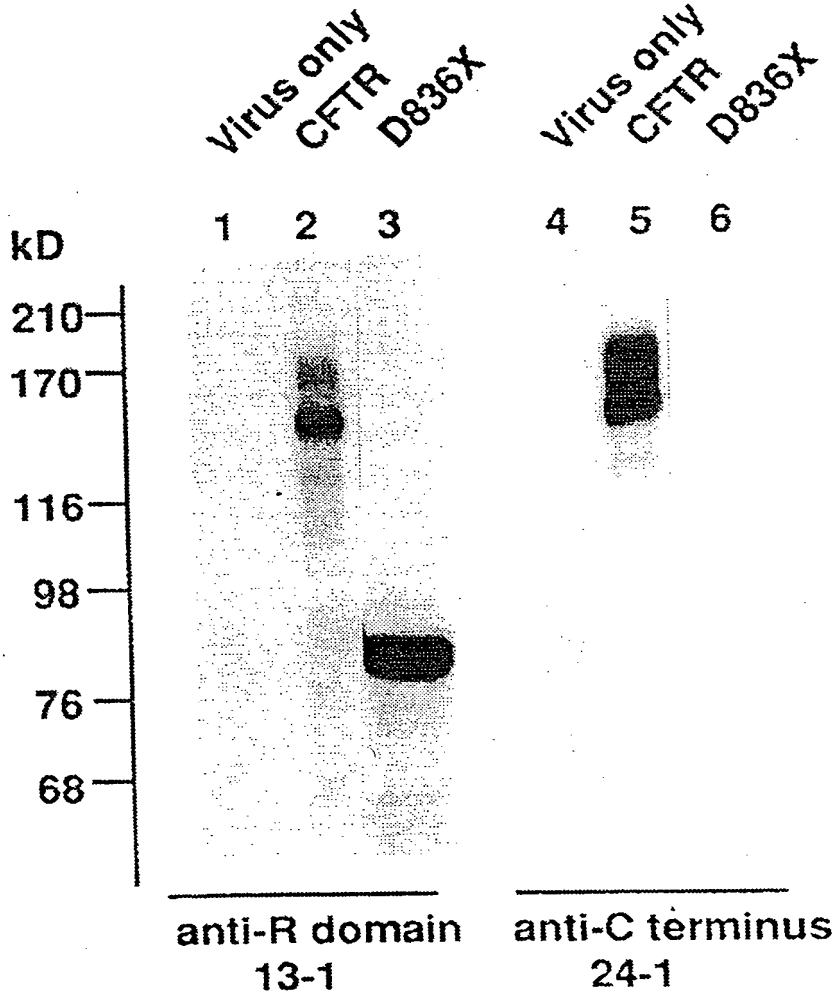
Virus only
CFTR
D836X
Virus only
CFTR
D836X
1 2 3 4 5 6
kD
210
170
116
98
76
68
anti-R domain
13-1
anti-C terminus
24-1

FIG. 1

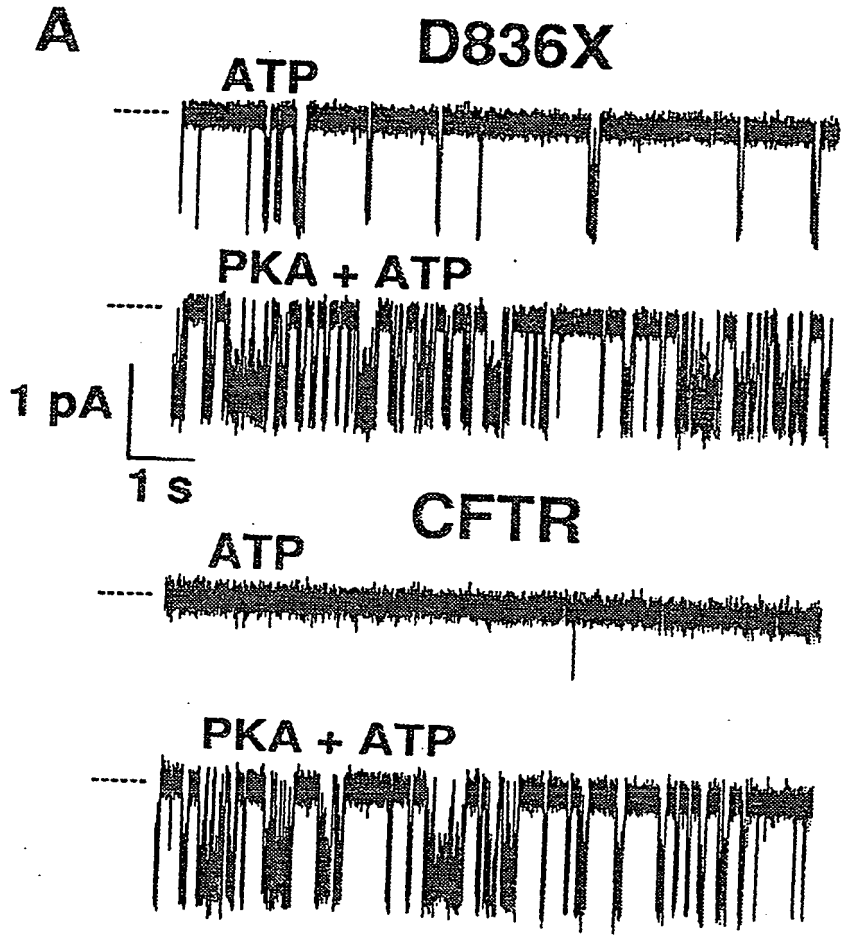
A
D836X
ATP
PKA + ATP
1 pA
1 s
CFTR
ATP
PKA + ATP

FIG. 2A

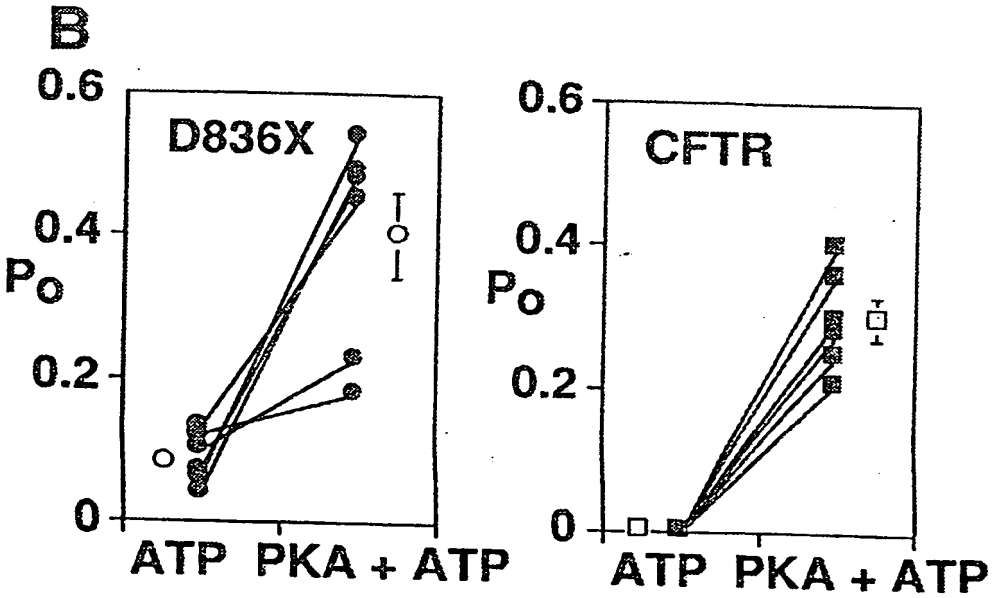
B
0.6
0.4
0.2
0
Po
D836X
ATP
PKA + ATP
CFTR

FIG. 2B

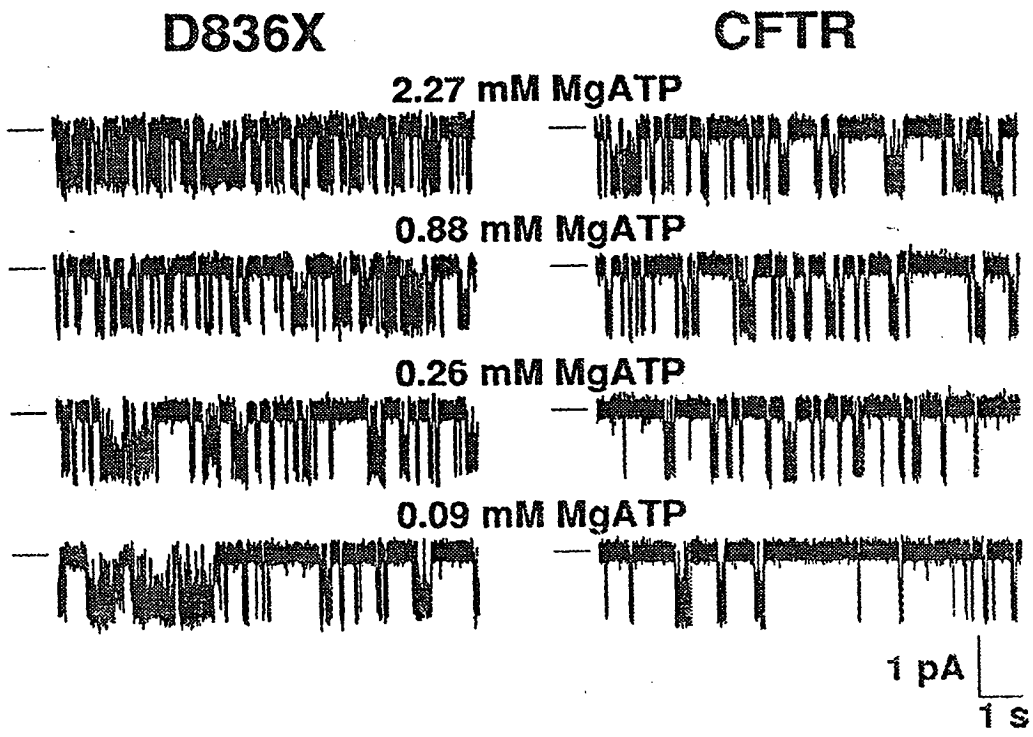

FIG. 3A

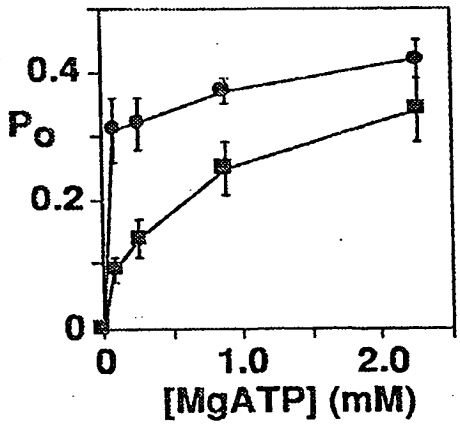

FIG. 3B

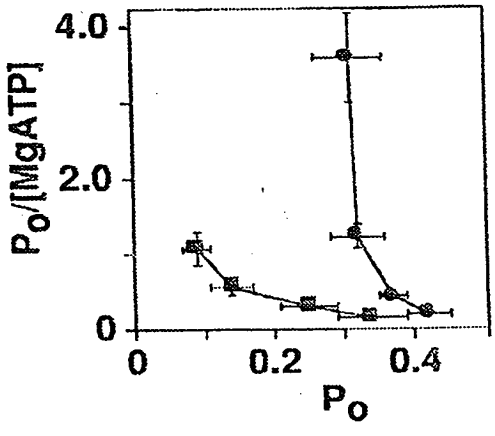

FIG. 3C

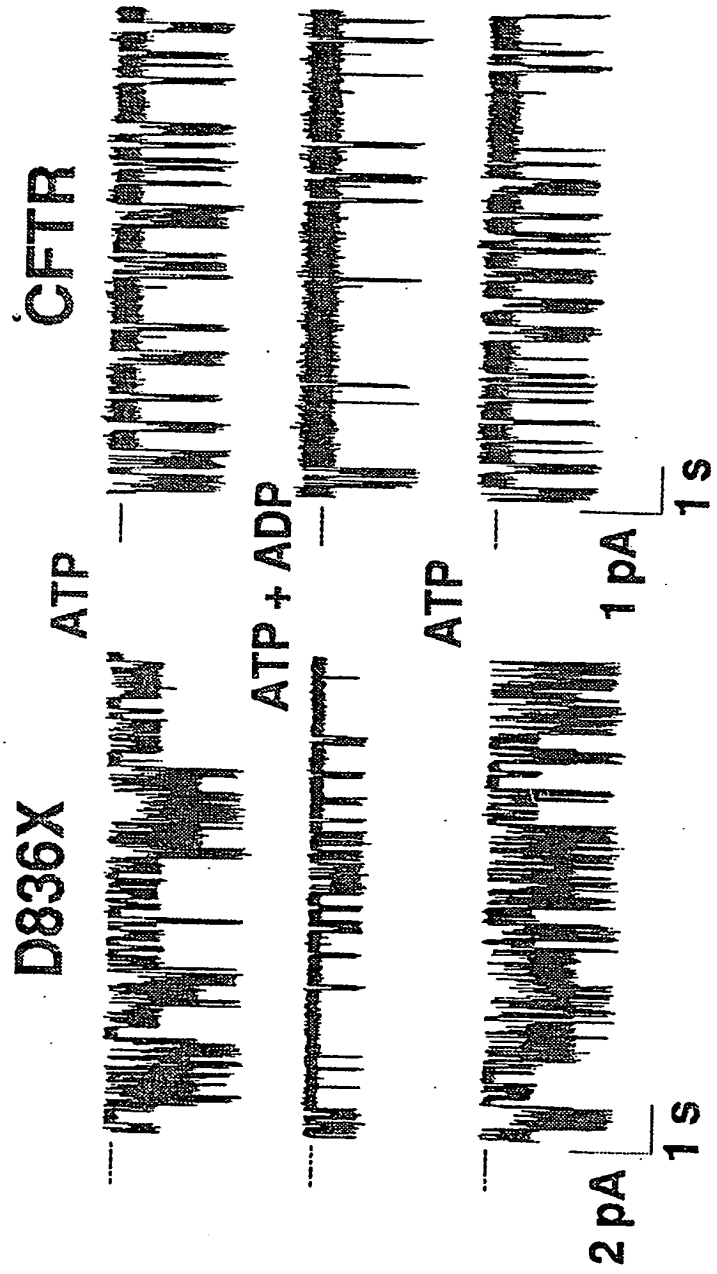
D836X
CFTR
ATP
ATP + ADP
ATP
1 pA
1 s
2 pA
1 s

FIG. 4A

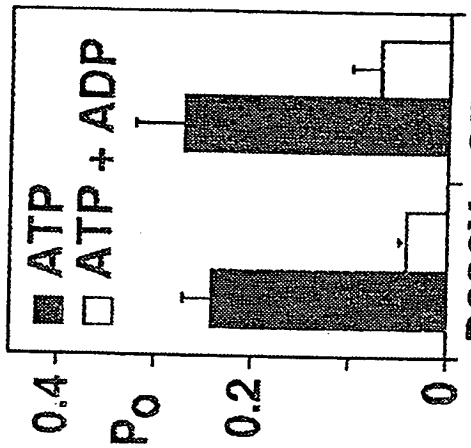
ATP
ATP + ADP
0.4
P_o
0.2
0
D836X CFTR

FIG. 4B

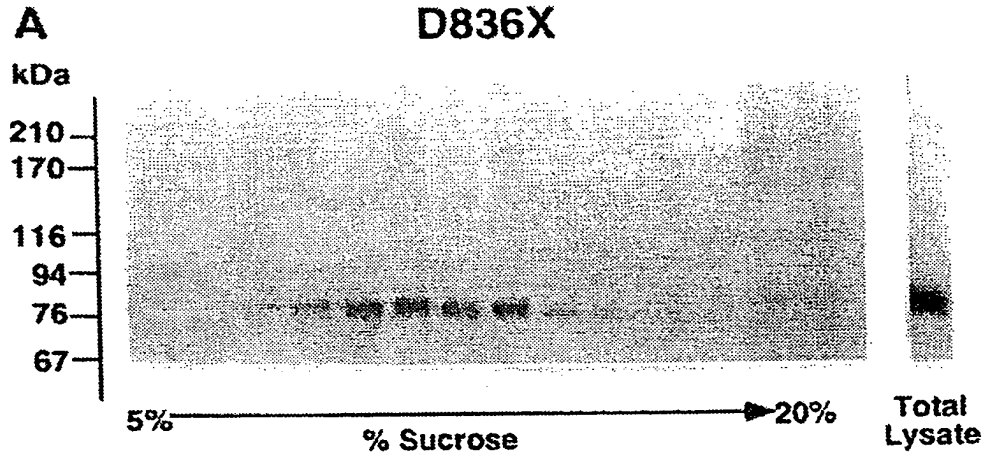

FIG. 5A

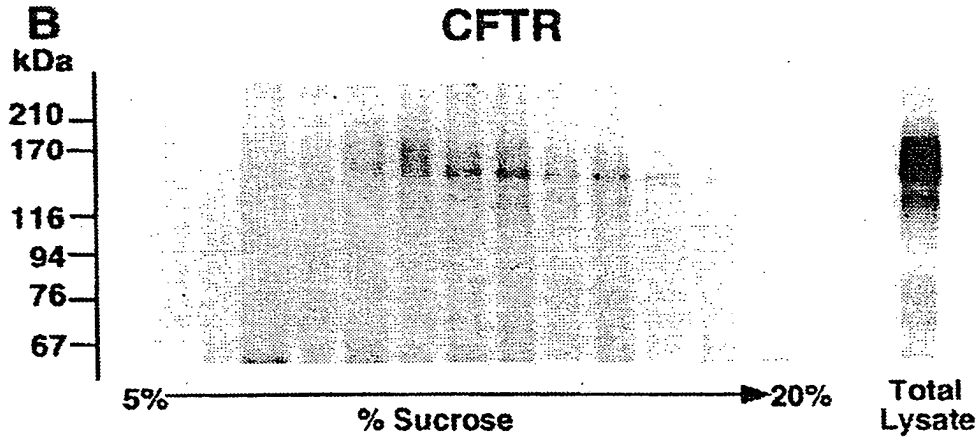

FIG. 5B

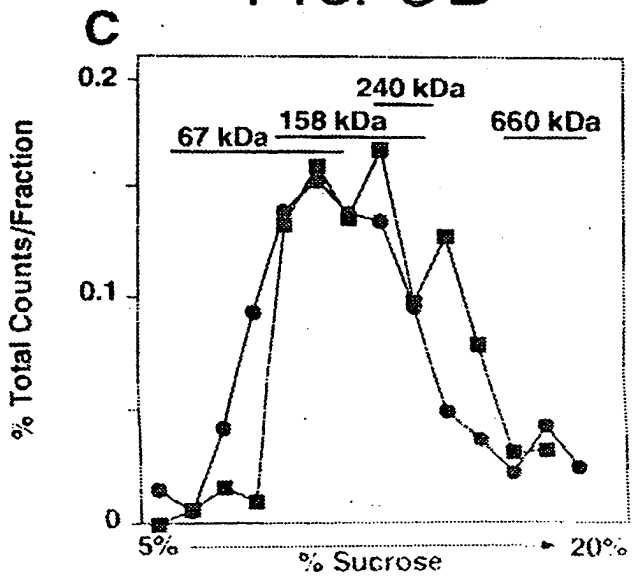

FIG. 5C

INTERNATIONAL SEARCH REPORT

[redacted]nal Application No
[redacted]T/US 95/03680

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 A61K48/00 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 10734 (HSC RESEARCH DEVELOPMENT CORPORATION) 25 July 1991 * Summary of the invention; p. 86, compound (xiii);	1-4, 6-16, 18-22
A	WO,A,92 05273 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 2 April 1992	
A	WO,A,93 03709 (VICAL, INC.) 4 March 1993	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 1995	04.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hermann, R

Form PCT/ISA/210 (second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9110734	25-07-91	CA-A-	2020817	11-01-92
		AU-A-	7060591	05-08-91
WO-A-9205273	02-04-92	US-A-	5240846	31-08-93
		CA-A-	2091882	19-03-92
		EP-A-	0549691	07-07-93
		JP-T-	6504188	19-05-94
WO-A-9303709	04-03-93	AU-A-	2143992	16-03-93
		CA-A-	2115364	04-03-93
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		JP-T-	6510036	10-11-94